

G6PD suppresses thrombopoiesis via interfering with TMOD3-mediated actin dynamics

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Dai

Thank you for the transfer of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are interesting and that the conclusions are overall supported by the data presented but they also raise a number of concerns and have suggestions on how to further strengthen the data; particularly, referees 2 and 3 requested greater characterization of actin distribution.

I agree with referee 1 (point 4) that increasing the cohort of human patients would strengthen the study. In case you have further data at hand, you are encouraged to include these, but it is not essential to increase sample size for publication in EMBO Reports. Similar considerations apply to the potential effects on platelet biogenesis in females, which should be discussed, but, again, the addition of further experimental data for this point is not required.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO Reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (February 1st). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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- 2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages <https://www.embopress.org/page/journal/14693178/authorguide> for more info on how to prepare your figures.
- 3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As

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(<<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>>)

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2' etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

<<https://www.embopress.org/page/journal/14693178/authorguide#expandedview>>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please include a dedicated "Data Availability" section at the end of the Methods (suggested wording: "The [structural coordinates | microarray | mass spectrometry] data from this publication have been deposited to the [name of the database] database [URL] and assigned the identifier [accession | permalink | hashtag]."). Should this not apply, this should still be stated as "This study includes no data deposited in external repositories." (see <<https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>>).

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10) Figure legends and data quantification:

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the EXACT p-values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:

<https://www.embopress.org/page/journal/14693178/authorguide#figureformat>

- Please also include scale bars in all microscopy images and define their size in the legend, not the image.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as

follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <<https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>>.

12) All Materials and Methods need to be described in the main text using our 'Structured Methods' format. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods, ideally using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs. Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines:

<https://www.embopress.org/page/journal/14693178/authorguide#structuredmethods>.

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<https://www.embopress.org/doi/10.15252/msb.20178071>.

13) As part of the EMBO publication's Transparent Editorial Process, EMBO Reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Kurt Weir
Editor
EMBO Reports

Referee #1:

This is a well-written manuscript, and the study addresses a topic of potential interest and significance. This study provides novel insight into the role of G6PD deficiency in platelet biogenesis, specifically through actin cytoskeletal remodelling mediated by TMOD3 localization, which is modulated by the G6PD-TMOD3 interaction in an NADP⁺-dependent manner. The measurements presented are comprehensive, and, overall, the experiments appear to have been conducted rigorously. However, there are several issues that need to be addressed, which may require additional experiments to strengthen the conclusions. The key points are as follows:

1. Since G6PD deficiency is X-linked and predominantly affects males, and this study includes only male subjects, it would be helpful if the authors clarified whether platelet biogenesis is similarly affected in females, either through data or discussion, as this is important for interpreting the findings.
2. While the primary focus of this manuscript is on platelet biogenesis in the context of G6PD deficiency, the authors also report a significant reduction in hemoglobin levels ($p = 0.0022$) in affected patients. This finding is not discussed further. Authors shall discuss the potential implications of decreased hemoglobin for platelet biogenesis in G6PD-deficient individuals.
3. In humans, mean platelet volume appears to be affected by G6PD deficiency, whereas this effect is not observed in knockout mice. Could the authors provide a possible explanation for this observation?
4. The study includes only 10 male patients, which is a relatively small sample size. The authors are encouraged to either increase the sample size or acknowledge this limitation in the manuscript.
5. The manuscript does not specify which methodology was used to measure the mice haematological parameters. This shall be included.
6. Details regarding the experimental procedures are incomplete. Specifically, the anticoagulant used for collecting murine blood for whole blood flow cytometry should be stated, and the protocol for isolating megakaryocytes from bone marrow-including subsequent histology and flow cytometry-is missing, as only the fetal liver method is described.
7. In Figure 2C, the expression of CD41 appears to be very low, whereas CD61 expression is high in megakaryocytes. As CD41

is also known to be expressed on megakaryocytes, could the authors provide a brief discussion or explanation for this observation.

8. In Section 2.5 and Figure 4F, G6P levels are reported to be significantly decreased in G6pd^{-/-} mice. The authors should discuss this finding, as G6P is an upstream substrate of G6P dehydrogenase. Additionally, it is important to clarify whether glucose levels were measured in these knockout mice.

9. The impact of the peptide GC2P2 could be further evaluated by assessing platelet functional parameters to determine whether it has any non-specific or off-target effects, including potential induction of platelet activation, aggregation, or changes in other hematological parameters.

10. The authors have presented a well-designed set of experiments demonstrating the involvement of TMOD3 in actin rearrangement with G6PD. However, it would be valuable if the authors could also discuss the potential roles of other components that may contribute to this process.

Minor Comments

1. On page 8, line 137, the text refers to Figures 2G and 2H; however, Figure 2H is not present. Please correct this reference.

2. In the Discussion section (page 14, line 286), the word "further" appears twice consecutively. Kindly revise to eliminate the duplication.

Referee #2:

The manuscript by Xin and colleagues examines the regulation of platelet production by studying the function of glucose-6-phosphate dehydrogenase (G6PD) beyond glucose metabolism. Using G6PD-deficient mouse model combined with co-immunoprecipitation assay and mass spectrometry, the authors report a new interaction between G6PD and actin-binding capping protein TMOD3 that limits proplatelet formation in vitro and platelet production in vivo.

The data show that disrupting the G6PD-TMOD3 interaction, either through conditional knockout or by targeting the key G6PD-TMOD3 interaction mediator NADPH⁺, leads to a notable increase in proplatelet formation and circulating platelet numbers. In addition, the authors developed a peptide to encourage G6PD-TMOD3 binding, which decreased platelet formation.

The identification of the G6PD-TMOD3 interaction as a key regulator of thrombopoiesis is a significant finding. A critical question that follows from this observation is whether the platelets produced are functionally competent. Therefore, a thorough analysis of the platelet phenotype is an essential addition needed to support the manuscript's conclusions.

Major comments

1. Significant changes reported in proplatelet formation strongly suggest alterations in the development of the demarcation membrane system (DMS). The current cytoskeletal staining is insufficient to confirm this.

In light of the potential impact on protein transport and the reported abnormalities in a related TMOD3 deficiency model (Sui et al., 2015), the authors should provide supplementary data in support of their conclusions concerning the modification of the mechanism of platelet biogenesis.

- a) DMS structure; MK maturation stage (via TEM or DMS analyse with specific markers by immunofluorescence); MK size.
- b) Number and distribution of granules along proplatelets and in tips;
- c) The characterisation of proplatelet extensions requires a more detailed analysis of branching, tip diameter and shaft thickness, as they sometimes appear thin and sometimes thick in the images.

2. A deeper analysis of the platelets is required :

- a) Platelet morphology; integrity of the marginal band and determination of the presence of granules
- b) A more in-depth characterisation of platelet functionality is required for both the KO model and platelets after peptide treatment. Beyond thrombin activation, the functionality of platelets with weaker agonists (e.g. ADP and CRP) should be investigated. MFI is used to present platelet activation. This analysis must be supplemented by presenting the percentage of pre-activated platelets in the absence of stimuli vs. activated platelets in the presence of agonists. Gating strategies and dot plots should be provided.
- c) Collagen aggregation shows an increase in lag time. What could explain this? Have you measured the expression of different surface glycoproteins, including GPIIb/IIIa?

3. As the main conclusions of the article are based on actin dynamics, it is essential to provide the following additional measurements:

- a) F-actin/tubulin and F-actin cytoplasm/cortex ratios;
- b) Quantification of nodule intensity. For example, in Figure 3B, some platelets in the G6PD KO have nodules and some do not, particularly the spread ones. What is the proportion of each type?
- c) In Figure 3A and Figure 5H of the KO, the nodules appear larger and/or more fluorescent when zoomed in. Could you quantify this?

4. What about the addition of NADP to platelets from WT vs KO mice in Fig. 6E?

This is only shown on human platelets.

5. Some results sometimes seem a little inconsistent and may undermine the reliability of the general conclusions:

- a) Fig. 2E vs Fig. 3A, we do not have the same view of the proplatelets at all; in Figure 2E they are thin, while in 3A they are very large and misshaped
- b) Fig. 6F: data vs quantification
- c) Figure 2B suggests that G6pd Δ/Δ megakaryocytes exhibit ectopic platelet release. There is pink staining in the medullary space. Could you confirm this using immunofluorescence or TEM?
- d) Could you provide the number of elements counted per field? Indeed, it is noted $n = x$, but how many cells are there per field; for platelets, proplatelets, MK/mm², etc.? This information would provide a clearer statistical representation.
- e) I do not understand the analysis in Figure 2C. Could you outline the full gating strategy? Could you also explain why more than 60% of the elements are CD41+ rather than CD42+?

Minor comments :

- 1. Authors should explain the observed populations in Fig. 2C
- 2. Could there be any possible effects of elevated NADP+ independent of G6PD-TMOD3 axis, as Fig. 6F suggests a decreasing trend for the KO line.
- 3. Figure 2H was mentioned several times, but the submitted manuscript does not contain such panel
- 4. Taking in account differences in the number of reticulated platelets that are known to be larger, could the differences in spreading area in Fig. 3B be the result of initial larger size of platelets?
- 5. Fig. 5D needs quantification to support the conclusions
- 6. Figure 5G would benefit from zoom image as in Fig. 5H and Fig. 6C
- 7. Figure 5H suggest polarization of the TMOD3 signal in G6pd Δ/Δ megakaryocytes. Is it representative of the whole population? Authors should address this point
- 8. Add a figure number to each one

Referee #3:

In their paper "G6PD suppresses thrombopoiesis via interfering with TMOD3-mediated actin dynamics", Xin et al. describe a novel, non-canonical function of G6PD in platelet formation. The authors demonstrate interactions between G6PD and TMOD3, thereby regulating actin dynamics. Their investigation combines multiple approaches: a G6PD knockout mouse model, several complementary cell line models using transfection or transduction, and a pharmacological strategy with a peptide. Notably, the study is introduced with patient data, which strengthens the clinical relevance of the topic. Overall, the article is of great interest, although additional experiments are required to consolidate some of the conclusions.

My detailed comments are provided below, following the order of the manuscript:

Page 5, line 81: please correct to G6PD-deficient.

Page 6:

In Table 1, the statistical analyses performed should be described.

For platelet count experiments, the sex and age of mice should be indicated.

Platelet aggregation and secretion assays should be repeated with different agonist concentrations to confirm the absence of effect of G6PD deletion.

Pages 7-8:

Line 130/137: Figure 2h is missing.

Line 133: the section title should be revised. The study does not demonstrate "accelerated" actin remodeling; only time-lapse or kinetic analyses could support such a claim.

Line 137: "could" should be used instead of a direct causal statement, as there is no definitive link between G6PD deficiency and altered actin dynamics.

Figure 3A / line 145: quantification of the spatial distribution of F-actin is required. Measurements of F-actin polymerization should also be provided in both cell lines and platelets. Without kinetic data, the conclusion should remain cautious with respect

to the suggested "acceleration."

Page 9, line 163: proteomic database comparisons between Ig control and specific IgG are needed to validate signal specificity.

Figure 5C: quantification and n values are missing.

Figures 6B-6D: authors should describe how relative protein levels were calculated.

Peptide experiments:

A negative control (e.g., scrambled peptide) is required in the biological assays.

It remains unclear whether the peptide promotes the interaction between G6PD and TMOD3, or instead acts as a dominant-negative inhibitor. This issue must be clarified experimentally and conceptually.

Page 19, line 344: replace neonatal platelets with immature platelets, IPF, or reticulated platelets.

Page 28: the section on statistical analyses should be expanded, specifying the tests used and clarifying the graphical representations.

Referee #1:

1. Since G6PD deficiency is X-linked and predominantly affects males, and this study includes only male subjects, it would be helpful if the authors clarified whether platelet biogenesis is similarly affected in females, either through data or discussion, as this is important for interpreting the findings.

We thank the reviewer for raising this important point. To address this comment, we collected and analyzed complete blood count (CBC) data from 7 female patients with G6PD deficiency. Comparative analysis with 30 age-matched healthy female controls revealed that platelet counts in female patients exhibited a trend (Figure 1A, new Appendix Figure S1A; 232.7 ± 38.53 vs 270.4 ± 29.67 , $p = 0.0208$) similar to that observed in male patients. We hope that this additional information helps clarify that platelet biogenesis is similarly affected in females and enhances the interpretation of our findings. We appreciate the reviewer's constructive suggestion.

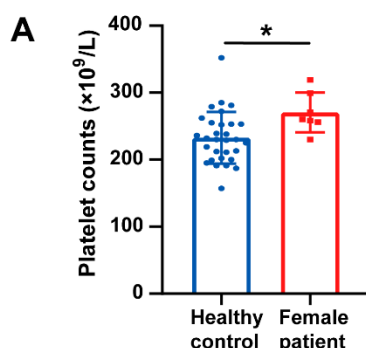
Fig. R1

Figure R1. (A) Platelet count of female healthy controls and G6PD-deficient patients ($n=30$ vs $n=7$, $*P<0.05$). All the enrolled subjects aged 18~40 years old. G6PD activity of the patient group: 1.929 ± 0.822 IU/g Hb.

Page 6, lines 102-104: “Statistical analysis revealed that the average platelet counts in 17 G6PD-deficient male patients was ~25% higher than normal controls (Table 1) and 6 female patients presented a similar trend (Appendix Fig. S1A), suggesting a regulatory role of G6PD in the platelet biology.”

2. While the primary focus of this manuscript is on platelet biogenesis in the context of G6PD deficiency, the authors also report a significant reduction in hemoglobin levels ($p = 0.0022$) in affected patients. This finding is not discussed further. Authors shall discuss the potential implications of decreased hemoglobin for platelet biogenesis in G6PD-deficient individuals.

We thank the reviewer for raising this important point. In response, we have added a discussion of this finding in the revised manuscript (page 14, lines 277–285).

Page 14, lines 277–285: “In patients with G6PD deficiency, we observed an approximately 8% reduction in hemoglobin levels (Table 1). We speculate that this is a concomitant manifestation of G6PD deficiency, as the condition is known to shorten erythrocytic lifespan (Luzzatto et al., 2020). Consistently, we also noted an approximately 6% decrease in red blood cell counts. It remains unclear whether the increased platelet counts are in part attributable to reduced hemoglobin levels, which may be accompanied by circulatory changes such as reduced oxygen-carrying capacity (Badireddy & Baradhi, 2025). Nevertheless, based on the finding in platelet specific G6PD-knockout murine model, we conclude that G6PD deficiency itself independently contributes to the increased platelet counts.”

3. In humans, mean platelet volume appears to be affected by G6PD deficiency, whereas this effect is not observed in knockout mice. Could the authors provide a possible explanation for this observation?

We thank the reviewer for this insightful question, which has also puzzled us. Although we do not currently have experimental data that directly address this discrepancy, we would like to offer several possible explanations for this question. First, it is important to note that, unlike the megakaryocyte/platelet-specific G6PD knockout mice used in our study, patients with G6PD deficiency carry the mutation in all cell types, which may be the origin of the observed difference. Megakaryocyte platelet production is known to be closely associated with the bone marrow microenvironment, including matrix stiffness, cellular components, metabolites, and

various factors that can influence megakaryocyte differentiation (Andrew P Stone et al, 2022; PMID:34587234). Although direct evidence is lacking, given the important role of G6PD in oxidative stress, we suspect that G6PD deficiency in bone marrow cells of patients may alter the bone marrow microenvironment, thereby affecting the volume of the platelets ultimately produced. This represents an intriguing direction that we plan to explore in future studies. Additionally, numerous reports have shown that mean platelet volume (MPV) is decreased in chronic inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis, and returns to normal upon treatment, suggesting a relationship between inflammation and MPV (Armen Yuri Gasparyan et al, 2011; PMID:21247392). Since G6PD deficiency is known to trigger inflammation, we speculate that this may also contribute to the reduced MPV observed in G6PD-deficient patients. These are our current thoughts on this matter, and we would welcome any alternative insights the reviewer might offer.

4. The study includes only 10 male patients, which is a relatively small sample size. The authors are encouraged to either increase the sample size or acknowledge this limitation in the manuscript.

We thank the reviewer for this constructive comment regarding the sample size. In response, we have increased the number of male patients with G6PD deficiency from 10 to 17, and the data are now presented in the updated Table 1 in the revised manuscript (page 43, lines 920-922). We believe that the expanded cohort further substantiates our findings. However, should the reviewer consider that the current sample size remains insufficient to fully support our conclusions, we are prepared to acknowledge this as a limitation in the Discussion section. We appreciate the reviewer's thoughtful guidance in improving the rigor of our study.

Before modification:

Table 1. Base line information of enrolled male healthy controls and G6PD-deficient patients.

Healthy control group (n=30)	G6PD-deficient group (n=10)	P
---------------------------------	--------------------------------	---

Age, years	31.2±5.83	29.3±7.62	0.414
G6PD activity*, IU/g Hb	/	0.55±1.11	/
Platelet count, ×10 ⁹ /L	220.3±39.82	286.7±66.24	P=0.001
Mean platelet volume, fL	9.89±0.95	9.14±0.70	0.0350
Red blood cell count, ×10 ¹² /L	4.96±0.27	4.69±0.83	0.0355
White blood cell count, ×10 ⁹ /L	6.14±1.54	6.53±2.26	0.5438
Red blood cell distribution width, %	12.96±0.59	12.69±1.21	0.6494
Hemoglobin concentration, g/L	151.7±8.52	139.8±13.45	0.0022

*The reference value for normal G6PD activity in the study was 2.8-7.3 IU/g Hb.

After modification:

Table 1. Base line information of enrolled male healthy controls and G6PD-deficient patients.

	Healthy control group (n=30)	G6PD-deficient group (n=17)	P
Age, years	31.2±5.83	29.82±6.48	0.4591
G6PD activity*, IU/g Hb	/	0.36±0.87	/
Platelet count, ×10 ⁹ /L	220.3±39.82	273.6±62.57	P=0.0009
Mean platelet volume, fL	9.89±0.95	8.89±0.76	0.0001
Red blood cell count, ×10 ¹² /L	4.96±0.27	4.64±0.76	0.0398
White blood cell count, ×10 ⁹ /L	6.14±1.54	6.32±1.76	0.7169
Red blood cell distribution width, %	12.96±0.59	12.78±1.33	0.6494
Hemoglobin concentration, g/L	151.7±8.52	139.9±13.03	0.0005

*The reference value for normal G6PD activity in the study was 2.8-7.3 IU/g Hb.

5. The manuscript does not specify which methodology was used to measure the mice haematological parameters. This shall be included.

6. Details regarding the experimental procedures are incomplete. Specifically, the anticoagulant used for collecting murine blood for whole blood flow cytometry should be

stated, and the protocol for isolating megakaryocytes from bone marrow-including subsequent histology and flow cytometry-is missing, as only the fetal liver method is described.

We thank the reviewer for pointing out these omissions. In response, we have now added a detailed description of the methodologies to the Methods section (page 22, lines 398–405; page 24–25, lines 452–469).

“Detection of mice haematological parameters

Using sodium citrate as an anticoagulant, whole blood was collected from murine fundus venous plexus. Platelet counts, along with MPV and P-LCR, were determined by Hematology Analyzer (Sysmex XN-1000™) based on the electrical impedance method.

Apoptotic and reticulated platelets assays in vitro

Fresh whole blood collected from murine fundus venous plexus was anticoagulated with sodium citrate and diluted 25-fold with Tyrode's buffer.

Immunohistochemical staining of murine bone marrow

For immunohistochemical analysis of megakaryocytes, femurs were isolated from anesthetized mice. After complete removal of adherent soft tissue by dissection, the femurs were fixed and decalcified, followed by paraffin embedding and sectioning. Immunohistochemical staining was subsequently carried out with a biotin-conjugated anti-CD42c antibody (1:100, LSBio) to label MK.

Isolation and flow cytometry staining of murine bone marrow cells

Mice were euthanized and fixed in a supine position on a dissection table. An incision was made along the longitudinal axis of the hind limb to expose the skin. The femoral and tibial joint ligaments were bluntly dissected to harvest the intact long bones of the lower limb. Bone marrow cavities were flushed with pre-chilled FACS buffer using a 2 mL syringe under pressure. The collected cell suspension was centrifuged at 800×g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 mL of ACK lysis buffer for erythrocyte lysis on ice for 10 minutes. The lysis reaction was terminated by adding 1 mL of FACS buffer. The

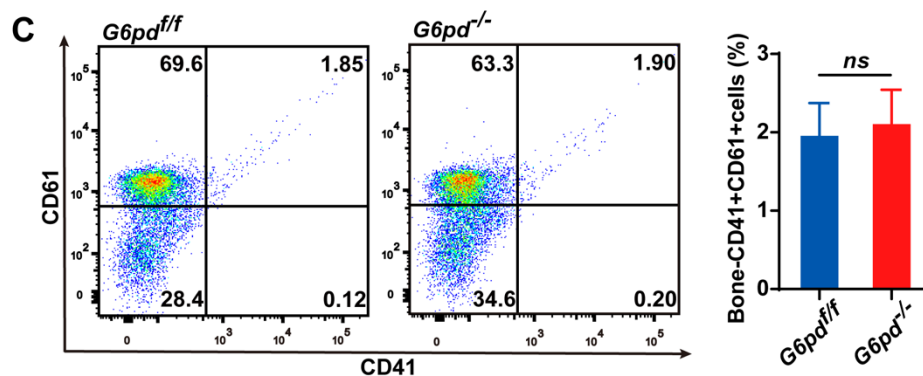
cell suspension was filtered through a 70 μ m cell strainer and resuspended in a final volume of 1 mL FACS buffer. Subsequently, 100 μ L of the sample was incubated with 1 μ L each of CD41 and CD61 antibodies for flow cytometry staining.”

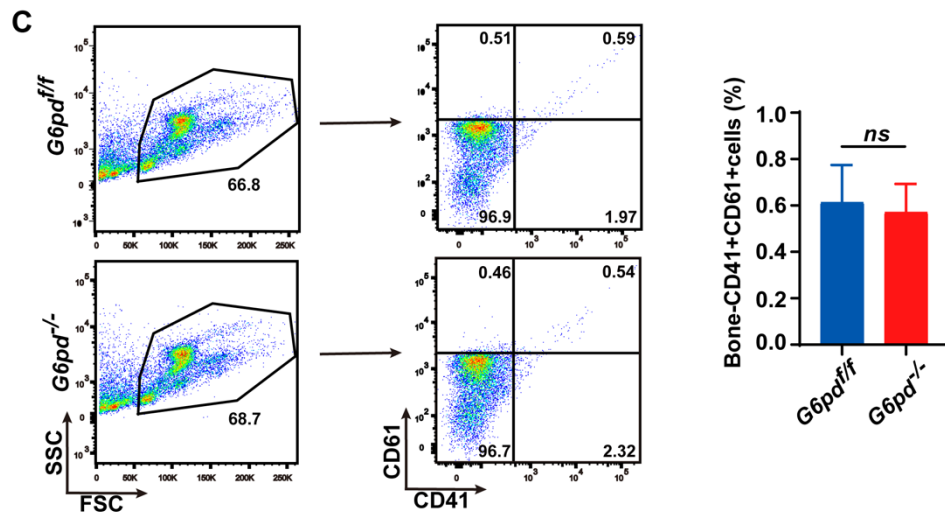
7. In Figure 2C, the expression of CD41 appears to be very low, whereas CD61 expression is high in megakaryocytes. As CD41 is also known to be expressed on megakaryocytes, could the authors provide a brief discussion or explanation for this observation.

We sincerely thank the reviewer for this critical observation regarding the expression of CD41 and CD61 in Figure 2C. We regret that an error was made during the initial data processing: the gating threshold for CD61 positivity was incorrectly set, and this mistake was not identified during our subsequent review. To address this, we have re-analyzed the raw data, replaced the image with the corrected version and added the gate strategy to the new version.

We apologize for this oversight and appreciate the reviewer’s careful scrutiny, which has helped us significantly improve the accuracy and clarity of our manuscript.

Before modification





8. In Section 2.5 and Figure 4F, G6P levels are reported to be significantly decreased in *G6pd^{-/-}* mice. The authors should discuss this finding, as G6P is an upstream substrate of G6P dehydrogenase. Additionally, it is important to clarify whether glucose levels were measured in these knockout mice.

We thank the reviewer for this insightful comment regarding the decreased G6P levels in *G6pd^{-/-}* mice and the important question about glucose measurement. We regret that the raw metabolomics data provided by the external company did not include glucose measurements. However, we fully agree with the reviewer that these data are essential.

To address this, we performed additional experiments using freshly isolated washed platelets from mice. Platelet pellets were lysed, and glucose levels were measured in equal numbers of platelets using an automated biochemical analyzer. The results are now presented in **Fig. R2** (new **Appendix Fig. S7A**). As shown, glucose levels in G6PD-deficient platelets exhibited a decreased trend compared to controls, suggesting that the reduction in G6P likely originates from a decreased availability of its precursor, glucose. We have added this discussion to the revised manuscript (page 14, lines 287–307). We appreciate the reviewer's insightful suggestion, which has prompted us to strengthen our mechanistic interpretation and improve the completeness of our data.

Fig. R2

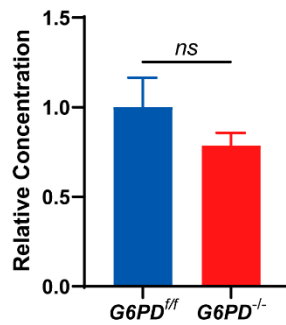


Figure R2. (A) *G6pd^{f/f}* and *G6pd^{-/-}* platelets were isolated and pelleted after washing, with equal cell numbers (3×10^8) used for each sample. The pellets were lysed in RIPA buffer, and glucose levels were measured using an automated biochemical analyzer. Statistical analysis of relative glucose content is shown in the figure (n=3).

Page 14, lines 287–307: “Glucose metabolism is the primary source of energy production and has been reported to play an essential role in platelet biology (Fidler et al., 2017). PPP is a non-energy-donating pathway that runs in parallel to glycolysis to produce NADPH and R5P (TeSlaa et al., 2023). Previous studies have indicated that in resting human platelets, approximately 37% of G6P enters PPP for metabolism (Sake et al., 2022), and an active PPP in human platelets was also observed in our study. G6PD is the rate-limiting enzyme in PPP, and its activity enormously determines the flux from glucose to PPP. Generally, a depressed PPP can result in an increased shunt to the ATP-donating glycolytic pathway, including glycolysis and the TCA cycle (Stincone et al., 2015; Zhong et al., 2021; Zodda et al., 2023). However, in G6PD-deficient murine platelets, we observed no significant alterations in the cellular levels of major glycolytic and TCA cycle intermediates. This raises an intriguing question: where did the “missing flux” go? Given the decreased levels of both G6P and glucose in G6PD-deficient platelets (Fig. 4F, and Appendix Fig. S7A), we propose the possible explanation: the blockade of PPP resulting from G6PD deficiency may feedback-inhibit glucose uptake, a phenomenon that has been reported (Grund et al, 2025). Furthermore, the cellular levels of NADPH and R5P remained largely unchanged in G6PD-deficient platelets, suggesting the existence of compensatory mechanisms through alternative metabolic pathways. However, our experimental

approach could not distinguish the specific sources. Nevertheless, one possible implication of these findings is that G6PD-mediated PPP flux is not indispensable in platelets, as metabolic plasticity and alternative routes may sustain the production of key metabolites under physiological conditions.”

9. The impact of the peptide GC2P2 could be further evaluated by assessing platelet functional parameters to determine whether it has any non-specific or off-target effects, including potential induction of platelet activation, aggregation, or changes in other hematological parameters.

To investigate the effect of the peptide on platelet function, platelets from *G6pd^{ff}* and *G6pd^{+/+}* mice were treated in vitro with control peptide or C-GC2P2. Following a 1-hour incubation of the peptide with washed platelets, aggregation and granule secretion were assessed upon stimulation with thrombin and collagen. The results, presented in **Fig. R3A-C**, revealed no differences between treatment groups. It must be acknowledged that due to the limited number of mice currently available and the relatively large quantity of platelets required for aggregation assays, only a single concentration of each agonist was repeatedly tested. Consequently, the aggregation data provide limited support for the conclusions drawn. However, in flow cytometric analyses of platelet granule secretion, a range of agonist concentrations representing different stimulation intensities was employed to evaluate the effects of peptide treatment on platelet function, and again, no differences were observed. Taken together, these findings suggested that the peptide exerted no non-specific effects on platelet activation.

Furthermore, in experiments involving peptide-treated mice, we evaluated mean platelet volume (MPV) and platelet-large cell ratio (P-LCR) at 48 hours post-injection—the time point at which the most pronounced difference in platelet counts was observed between control mice from the control peptide and C-GC2P2 groups. The results revealed no significant differences between groups (**Fig. R3D**), indicating that GC2P2 does not exert appreciable off-target effects on these platelet parameters.

These results have now been incorporated into the revised manuscript (new **Appendix Fig. S6A-D**; page 14, lines 273–275). We appreciate the reviewer’s constructive comment, which has helped us more comprehensively characterize the specificity of the peptide intervention.

page 14, lines 273–275: “Notably, no significant effects of C-GC2P2 were observed on platelet aggregation, granule release, or morphological parameters (Appendix Fig. S6A-D).”

Fig. R3

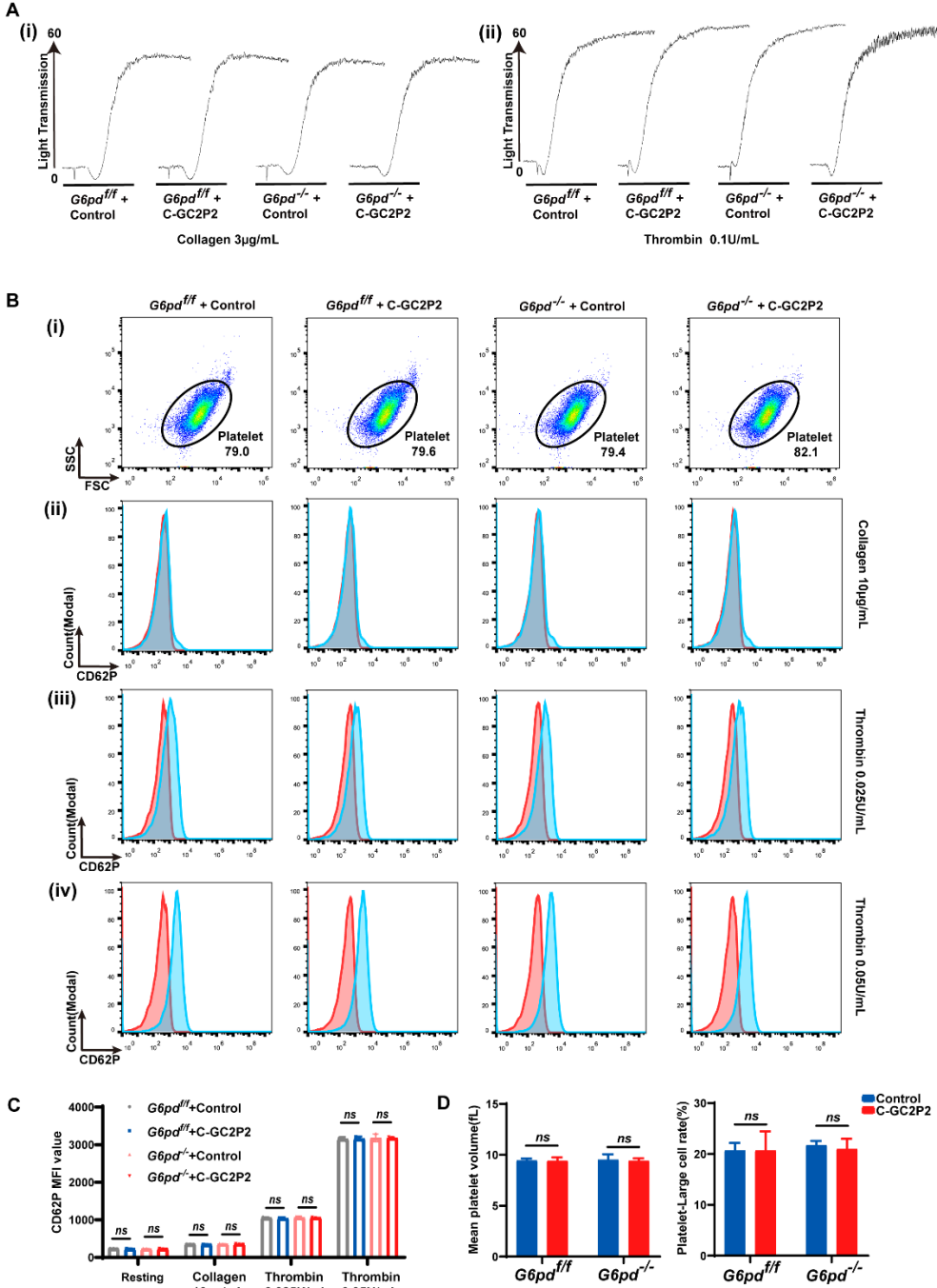


Figure R3. Peptide C-GC2P2 showed no impact on size, in-vitro aggregation or secretion of *G6pd^{fl/fl}* and *G6pd^{-/-}* platelets. (A) Aggregation of washed *G6pd^{fl/fl}* and *G6pd^{-/-}* platelets (treated with 50 µg/mL control peptide CPP-Scramble and peptide C-GC2P2) in response to collagen (3 µg/mL) and thrombin (0.1 U/mL). (B-C) Flow cytometry was used to detect the CD62P (P-selectin) exposure of washed *G6pd^{fl/fl}* and *G6pd^{-/-}* platelets (treated with control peptide CPP-Scramble and peptide C-GC2P2) stimulated with collagen (10 µg/mL) and thrombin (0.025 U/mL, 0.05U/mL). (B) The gating strategy is presented (i), along with overlaid histograms depicting platelet activation in response to various stimuli (ii-iv). In figure ii-iv, resting and activated platelets are shown in red and blue, respectively. (C) Platelet activation was assessed by determining the MFI of CD62P following exposure to different stimuli (n=3). (D) MPV and the P-LCR at 48 hours after peptide injection (i.e., Day 4) in both *G6pd^{fl/fl}* and *G6pd^{-/-}* mice (n=3).

10. The authors have presented a well-designed set of experiments demonstrating the involvement of TMOD3 in actin rearrangement with G6PD. However, it would be valuable if the authors could also discuss the potential roles of other components that may contribute to this process.

We thank the reviewer for this highly constructive suggestion. In response, we have added a discussion in the revised manuscript (page 16, lines 325–331) acknowledging that the interaction between G6PD and TMOD3 likely accounts for only part of the mechanism underlying the increased platelet counts observed in G6PD deficiency. Other components may also contribute to this process. We believe that this additional perspective provides a more comprehensive view of the molecular landscape involved and opens up interesting directions for future investigation.

Page 16, lines 325–331: “Notably, our proteomic analysis identified several additional candidates—including FYN-binding protein 1 (Spindler et al, 2018), MOB kinase activator (a regulator of the Hippo pathway) (Lorthongpanich et al, 2017), and AP-2 complex subunit beta (Hitchcock et al, 2008)—all of which have previously been implicated in platelet biogenesis, prompting that multiple signaling and cytoskeletal regulators may cooperate with or act in parallel to the G6PD–TMOD3 axis in modulating proplatelet formation.”

Minor Comments

1. On page 8, line 137, the text refers to Figures 2G and 2H; however, Figure 2H is not present. Please correct this reference.

2. In the Discussion section (page 14, line 286), the word "further" appears twice consecutively. Kindly revise to eliminate the duplication.

We thank the reviewer for pointing out these oversights. We have now corrected the citation "Fig. 2G, H" to "Fig. 2F, I, J" and delete one "further" in the revised manuscript (page 8, line 156; page 17, line 340). We apologize for this error and appreciate the reviewer's careful reading again.

Referee #2:

The manuscript by Xin and colleagues examines the regulation of platelet production by studying the function of glucose-6-phosphate dehydrogenase (G6PD) beyond glucose metabolism. Using G6PD-deficient mouse model combined with co-immunoprecipitation assay and mass spectrometry, the authors report a new interaction between G6PD and actin-binding capping protein TMOD3 that limits proplatelet formation in vitro and platelet production in vivo.

The data show that disrupting the G6PD-TMOD3 interaction, either through conditional knockout or by targeting the key G6PD-TMOD3 interaction mediator NADPH⁺, leads to a notable increase in proplatelet formation and circulating platelet numbers. In addition, the authors developed a peptide to encourage G6PD-TMOD3 binding, which decreased platelet formation.

The identification of the G6PD-TMOD3 interaction as a key regulator of thrombopoiesis is a significant finding. A critical question that follows from this observation is whether the platelets produced are functionally competent. Therefore, a thorough analysis of the platelet phenotype is an essential addition needed to support the manuscript's conclusions.

Major comments

1. Significant changes reported in proplatelet formation strongly suggest alterations in the development of the demarcation membrane system (DMS). The current cytoskeletal staining is insufficient to confirm this.

In light of the potential impact on protein transport and the reported abnormalities in a related *TMOD3* deficiency model (Sui et al., 2015), the authors should provide supplementary data in support of their conclusions concerning the modification of the mechanism of platelet biogenesis.

a) DMS structure; MK maturation stage (via TEM or DMS analyse with specific markers by immunofluorescence); MK size.

We sincerely thank the reviewer for this insightful and constructive suggestion. To figure out whether G6PD deficiency altered DMS structure, maturation stage and size of megakaryocytes, we have performed additional experiments and analysis using TPO-induced megakaryocytes *in vitro*. These data are present in Fig. R4 and included in the revised manuscript (new **Figure EV 2A-C; page 7-8, lines 134–142**). Megakaryocyte maturation staging followed the methodology of an article on *Blood* (Marc L Ellis et al, 2024; PMID: 37922495). The protocol of TEM sample preparation has also been included in the revised manuscript (**page 28, lines 530-544**). We are grateful to the reviewer for prompting us to strengthen this critical aspect of our mechanistic characterization; these additions have substantially improved the rigor and depth of our study.

Fig. R4

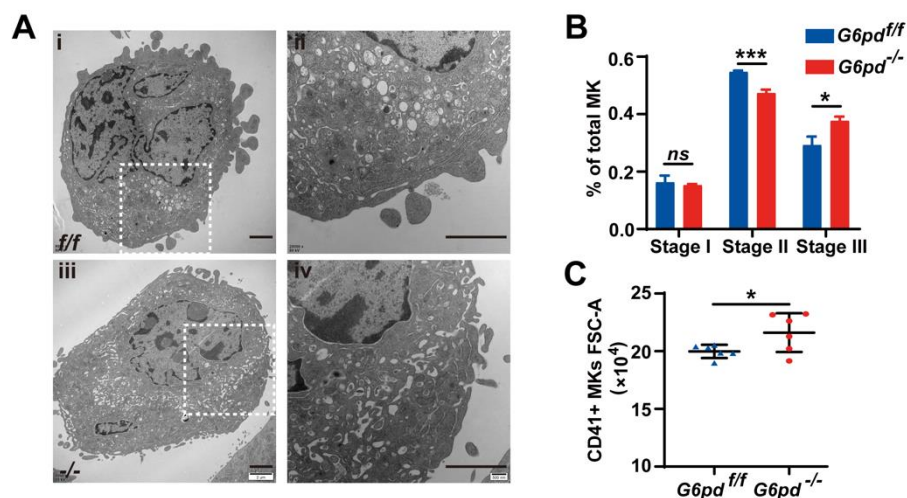


Figure R4. G6PD deficiency influenced demarcation membrane systems (DMS) formation of megakaryocyte. (A) *G6pd^{fl/fl}* and *G6pd^{-/-}* MKs derived from induced fetal liver cells were visualized by electron microscopy. Representative ultrastructural images (i and ii for *G6pd^{fl/fl}* MKs, iii and iv for *G6pd^{-/-}* MKs) were presented. Scale bars represented 2 μ m. (B) Megakaryocytes were categorized into three maturation stages based on ultrastructural features under the electron microscopy. Percentage of different stages of MKs from three group *G6pd^{fl/fl}* and *G6pd^{-/-}* fetal livers were analyzed (n=3, *P<0.05, ***P<0.001). Stage I corresponded to cells approximately 10 μ m in diameter with a large nucleus; Stage II to cells 10 to 20 μ m in diameter containing platelet-specific granules; and Stage III to mature MKs with well-developed DMS, distinct platelet territories, and a peripheral zone. (C) MK size analysis by flow cytometry using the forward scatter (FSC) value of CD41⁺ cells (MKs) derived from induced fetal liver cells.

lines 134–142: “We further examined the ultrastructure of MKs using transmission electron microscopy (TEM), which confirmed a more elaborate DMS network characterized by expanded internal membranes and more abundant membranous protrusions at the cell periphery in mature *G6pd^{-/-}* MKs (Fig. EV2A). And in *G6pd^{-/-}* MKs, the proportion of stage II ultrastructural maturation was significantly decreased, whereas that of stage III was markedly increased compared to controls (Fig. EV2B). Additionally, flow cytometric analysis demonstrated a significant increase in the size (forward scatter, FSC) of G6PD-deficient megakaryocytes (Fig. EV2C). These findings demonstrated that G6PD deficiency could lead to enhanced MK cytoplasmic maturation.”

b) Number and distribution of granules along proplatelets and in tips;

c) The characterisation of proplatelet extensions requires a more detailed analysis of branching, tip diameter and shaft thickness, as they sometimes appear thin and sometimes thick in the images.

We thank the reviewer for these insightful comments regarding the detailed characterization of proplatelet extensions and granule distribution. In response, we have performed a more comprehensive analysis following the methodology described in Sui et al., 2015 [PMID:

26051946], specifically examining the number and distribution of vWF-positive granules along proplatelets and in tips, as well as the branching, tip diameter, and shaft thickness of proplatelet extensions. Quantification of proplatelet branching has already been presented in **Fig. 2I** (page 8, line 147), demonstrating that G6PD-deficient megakaryocytes exhibit significantly more proplatelet tips. For the additional parameters, we found that neither the density nor the distribution of vWF-positive granules along the proplatelet shaft or within terminal buds differed between genotypes, nor were there significant changes in shaft thickness or tip diameter of individual proplatelet extensions. These new data are now presented in Fig. R5 and incorporated into the revised manuscript as **Fig. EV2D–H** (page 8, lines 148–151). Collectively, our analysis indicates that while G6PD deficiency promotes the initiation of proplatelet branches, it does not substantially affect the structural properties of the extensions once formed or the granular content along them. We are grateful to the reviewer for prompting this more detailed characterization, which has significantly strengthened our study.

Fig. R5

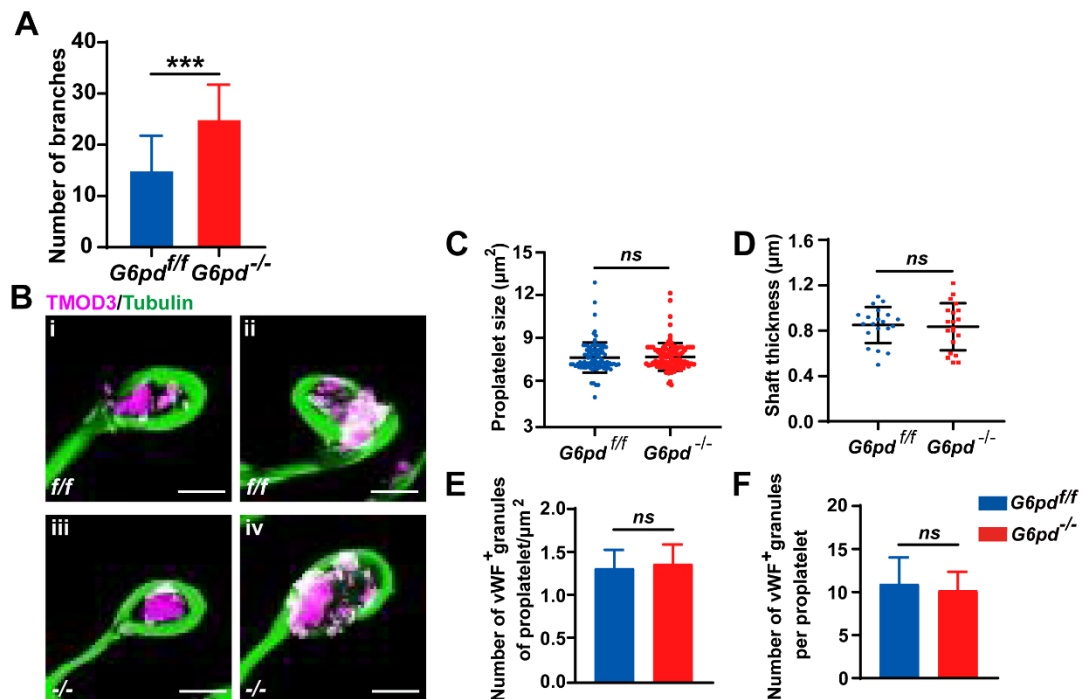


Figure R5. (A) Numbers of branches of per *G6pd^{f/f}* and *G6pd^{-/-}* PPF-MKs (n=15, ***P<0.001). (B) Representative high magnification images of vWF (purple) and tubulin (green) fluorescence in *G6pd^{f/f}* and *G6pd^{-/-}* proplatelets. Scale bars represented 2 μm . (C) Sizes of proplatelet buds produced by cultured MKs (n=100). (D) Thickness of

proplatelet shaft connected with buds (n=20). (E, F) The density and number of vWF⁺ granules in proplatelets.

2. A deeper analysis of the platelets is required :

a) Platelet morphology; integrity of the marginal band and determination of the presence of granules

To characterize platelet morphology, we performed TEM on washed platelets isolated from control and G6PD-deficient mice. As shown in Fig. R6 (now included as Fig. EV1E; page 8, lines 149–151 in the revised manuscript), the integrity of the marginal microtubule band was comparable between genotypes, and quantification of α -granules and dense granules per unit area revealed no significant differences. These results indicate that G6PD deficiency does not substantially affect the ultrastructural morphology or granule content of resting platelets.

Fig. R6

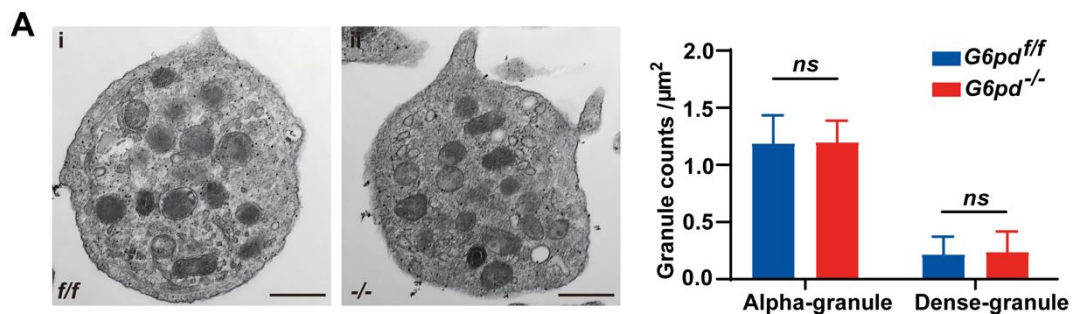


Fig. R6 (A) Representative ultrastructural images and granule analysis of washed *G6pd^{f/f}* and *G6pd^{-/-}* platelets visualized by electron microscopy (n=10). Scale bars represented 0.5 μ m.

b) A more in-depth characterisation of platelet functionality is required for both the KO model and platelets after peptide treatment. Beyond thrombin activation, the functionality of platelets with weaker agonists (e.g. ADP and CRP) should be investigated. MFI is used to present platelet activation. This analysis must be supplemented by presenting the percentage of pre-activated platelets in the absence of stimuli vs. activated platelets in the presence of

agonists. Gating strategies and dot plots should be provided.

To investigate the effect of the peptide on platelet function, platelets from *G6pd^{fl/fl}* and *G6pd^{+/-}* mice were treated in vitro with control peptide or C-GC2P2. Following a 1-hour incubation of the peptide with washed platelets, aggregation and granule secretion were assessed upon stimulation with thrombin and collagen. The results, presented in **Fig. R7A-C** (new **Appendix Fig. S6A-C**; **page 14, lines 273–275** in the revised manuscript), revealed no differences between treatment groups. It must be acknowledged that due to the limited number of mice currently available and the relatively large quantity of platelets required for aggregation assays, only a single concentration of each agonist was repeatedly tested.

We fully appreciate the reviewer's concern that G6PD deficiency might preferentially affect platelet responses under submaximal stimulation. However, we do not currently have access to CRP. Regarding ADP, we made multiple attempts using various sources of ADP to stimulate platelets, but consistently failed to induce detectable aggregation or granule secretion under our experimental conditions, likely due to insufficient agonist potency. Unfortunately, due to subsequent depletion of our mouse colony, we were unable to pursue additional optimization with ADP.

Consequently, the aggregation data provided limited support for the conclusions drawn. However, in flow cytometric analyses of platelet granule secretion (Fig R7C), a range of agonist concentrations representing different stimulation intensities was employed to evaluate the effects of peptide treatment on platelet function, and again, no differences were observed. Taken together, these findings suggested that the peptide exerted no non-specific effects on platelet activation.

Fig. R7

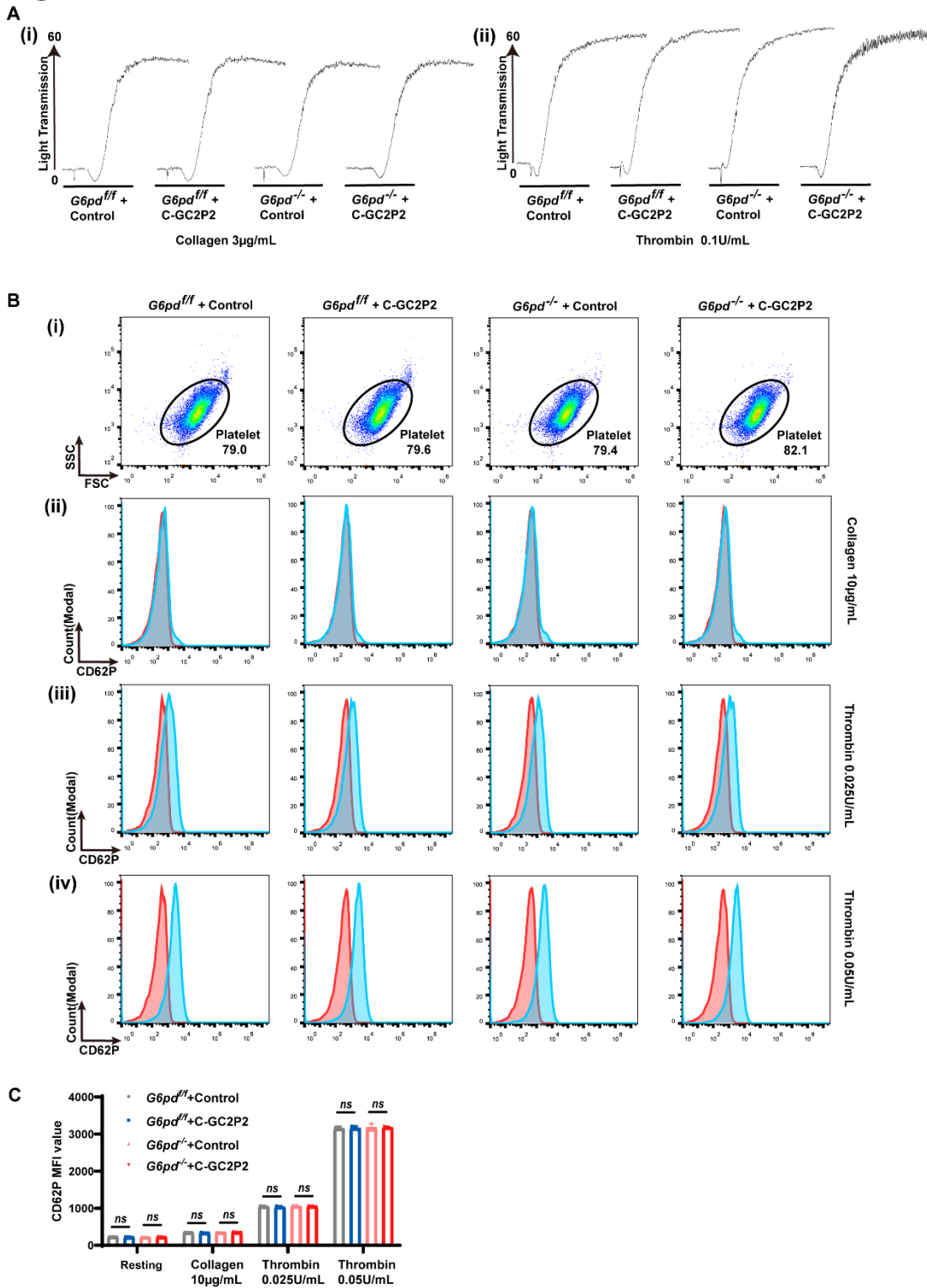


Figure R7. Peptide C-GC2P2 showed no impact on size, in-vitro aggregation or secretion of *G6pd^{fl/fl}* and *G6pd^{-/-}* platelets. (A) Aggregation of washed *G6pd^{fl/fl}* and *G6pd^{-/-}* platelets (treated with 50 μ g/mL control peptide CPP-Scramble and peptide C-GC2P2) in response to collagen (3 μ g/mL) and thrombin (0.1

U/mL). (B-C) Flow cytometry was used to detect the CD62P (P-selectin) exposure of washed *G6pd^{fl/fl}* and *G6pd^{-/-}* platelets (treated with control peptide CPP-Scramble and peptide C-GC2P2) stimulated with collagen (10 µg/mL) and thrombin (0.025 U/mL, 0.05U/mL). (B) The gating strategy is presented (i), along with overlaid histograms depicting platelet activation in response to various stimuli (ii-iv). In figure ii-iv, resting and activated platelets are shown in red and blue, respectively. (C) Platelet activation was assessed by determining the MFI of CD62P following exposure to different stimuli (n=3).

c) Collagen aggregation shows an increase in lag time. What could explain this? Have you measured the expression of different surface glycoproteins, including GPVI?

In our initial analysis, we focused primarily on the final aggregation rate (light transmission) and inadvertently did not select representative tracings that accurately reflected the lag time. To address this, we have now included aggregation results using different concentrations of collagen in the revised manuscript, and we have replaced the representative aggregation tracings with ones that better illustrate the lag phase. As shown in Fig. R8A (new **Fig. EV1B; page 6, lines 111-113** in the revised manuscript), the lag times for collagen-induced aggregation were comparable between G6PD-deficient and wild-type platelets under the conditions tested. Furthermore, as shown in Fig. R8B (new **Fig. EV1D; page 6, lines 111-113** in the revised manuscript), we have measured the expression of several major platelet surface glycoproteins, including CD41, CD42b, CD61, and GPVI, by flow cytometry. Our results demonstrate that G6PD deficiency did not significantly alter the surface levels of these glycoproteins. We appreciate the reviewer's careful scrutiny, which has prompted us to refine our data presentation and strengthen the rigor of our aggregation analyses.

Fig. R8

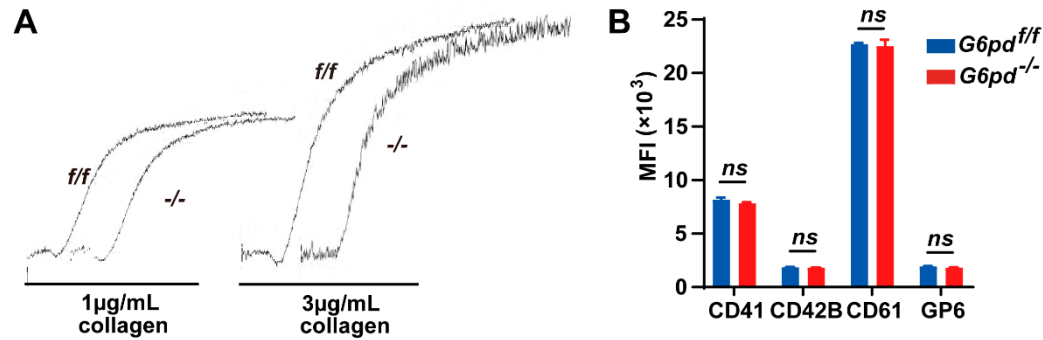


Figure R8. (A) Aggregation of washed *G6pd^{f/f}* and *G6pd^{-/-}* platelets in response to collagen (1 µg/mL, 3 µg/mL). **(B)** The expression of different surface glycoproteins of *G6pd^{f/f}* and *G6pd^{-/-}* platelets detected by flow cytometry (n=3).

3. As the main conclusions of the article are based on actin dynamics, it is essential to provide the following additional measurements:

a) F-actin/tubulin and F-actin cytoplasm/cortex ratios;

In response, we have performed a more comprehensive analysis of F-actin in proplatelet buds following the methodology described in Sui et al., 2015 [PMID: 26051946]. As shown in Fig. R9 (new Fig. EV2I-K; page 9, lines 161-162 in the revised manuscript), no significant alterations were observed in the F-actin content or distribution within the generated proplatelet buds.

Fig. R9

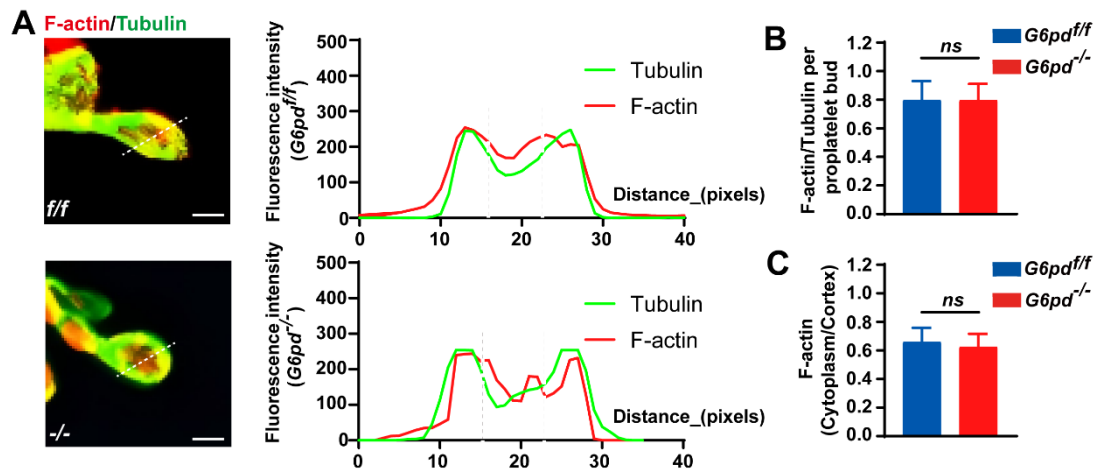


Figure R9. (A) Representative high magnification images and representative line scans of tubulin (green) and F-actin (red) fluorescence in *G6pd^{f/f}* and *G6pd^{-/-}* proplatelets. Scale bars represented 2 μ m. **(B)** Relative level of F-actin vs tubulin per proplatelet bud, calculated from fluorescence intensity ratios with Image J (n=20). **(C)** F-actin intensity of cytoplasm vs cortex in proplatelet bud (n=20), which were determined from intensities within ~ 0.75 μ m diameter circles placed over the edge or middle of the proplatelet, using Image J software.

b) Quantification of nodule intensity. For example, in Figure 3B, some platelets in the *G6PD* *KO* have nodules and some do not, particularly the spread ones. What is the proportion of each type?

Platelet spreading involves multiple stages of cytoskeletal reorganization, including the formation of actin nodules for adhesion, followed by the generation of filopodia, lamellipodia, and actin filaments for area expansion (S.D.J. CALAMINUS et al, 2008; PMID: 18761725). In response, we categorized platelets into distinct stages based on whether nodules or other actin structures predominated, and the statistical results are presented in Fig. R10 and in the revised manuscript (new Fig. 3B, C; page 9, lines 166-168 in the revised manuscript). The analysis revealed that within the same time frame, a significantly higher proportion of *G6PD*-deficient platelets had progressed to later spreading stages compared to controls, which is consistent with the observed differences in spreading area.

Fig. R10

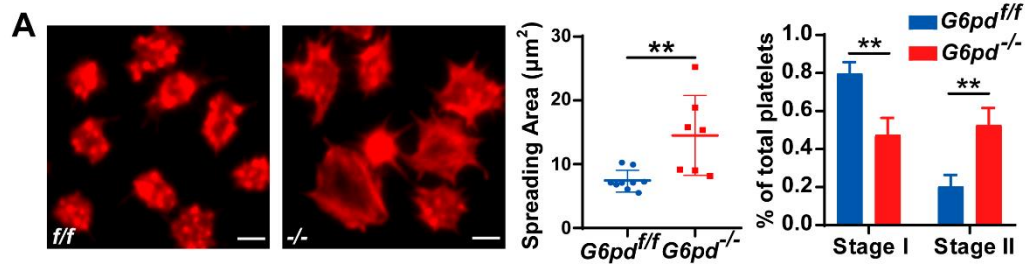


Fig. R10 (A) Representative immunofluorescence images of actin cytoskeleton in *G6pd^{f/f}* and *G6pd^{-/-}* murine platelets spreading on immobilized fibrinogen. Actin cytoskeleton in platelets was stained with rhodamine-labeled phalloidin. Statistical analysis of the platelets spreading areas from the two images was presented (n=9 vs n=7, **P<0.01). Scale bars represented 2 μm. Platelets were categorized into stages based on the predominance of nodules (Stage I) versus predominance of other actin structures (Stage II), and the percentage of platelets at different stages from three random fields captured under 63× oil immersion objective were presented (n=3, **P<0.01).

c) In Figure 3A and Figure 5H of the KO, the nodules appear larger and/or more fluorescent when zoomed in. Could you quantify this?

We thank the reviewer for this detailed observation. We would like to clarify that the cells shown in these two figures represent distinct stages of megakaryocyte maturation: original Figure 3A depicts megakaryocytes actively generating proplatelets (the proplatelet-formation stage), whereas original Figure 5H shows megakaryocytes at an earlier stage prior to proplatelet formation. Following the reviewer's suggestion, we quantified the actin nodules (podosomes) in megakaryocytes at the pre-proplatelet stage (as shown in Fig. 5H in the manuscript). Our analysis revealed that nodules in *G6pd^{-/-}* megakaryocytes were indeed larger compared to controls. These quantitative data are now presented in Fig. R11 and have been incorporated into the revised manuscript as new **Appendix Fig. S3C (page 11, line 215)**. However, we apologize that quantification of nodules in proplatelet-forming megakaryocytes in original Figure 3A proved technically difficult, particularly because actin filaments in *G6pd^{f/f}* megakaryocytes at this stage tend to form dense aggregates that are not amenable to reliable nodule counting.

Fig. R11

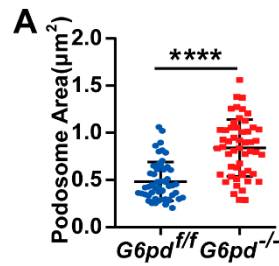


Fig. R11 (A) Statistical analysis of podosome area from Figure 5H in the revised manuscript (n=52).

4. What about the addition of NADP to platelets from WT vs KO mice in Fig. 6E?

This is only shown on human platelets.

In our previous experiments, we had already analyzed the effect of NADP⁺ on platelet spreading in both WT and KO mice. As shown in Fig. R12 (new Appendix Figure S4B; page 12, lines 242-244), NADP⁺ significantly inhibited the spreading of WT platelets, whereas no significant effect was observed in KO platelets.

Fig. R12

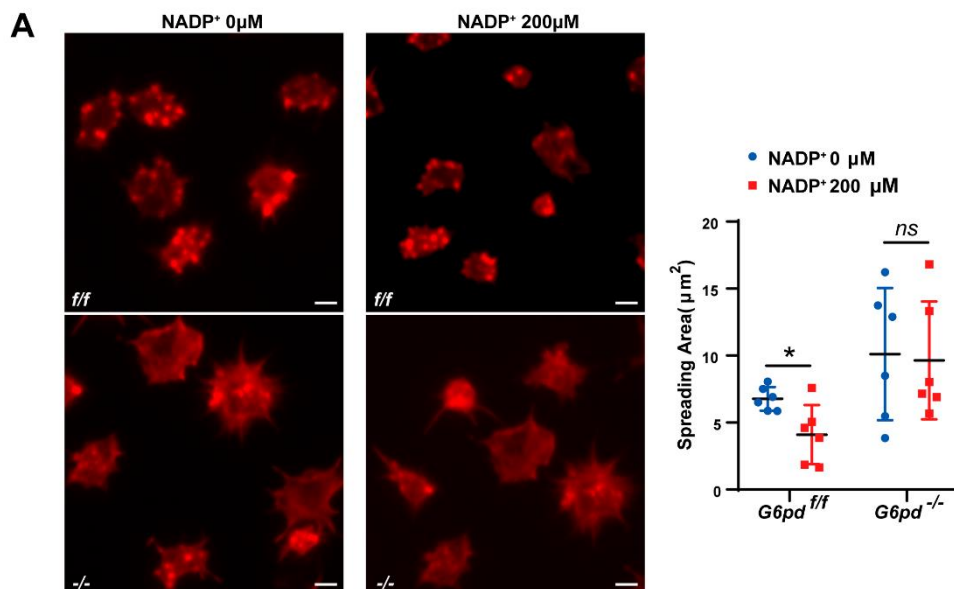


Fig. R12 (A) Immunofluorescence images of actin cytoskeleton in $G6pd^{f/f}$ and $G6pd^{-/-}$ murine platelets (untreated or

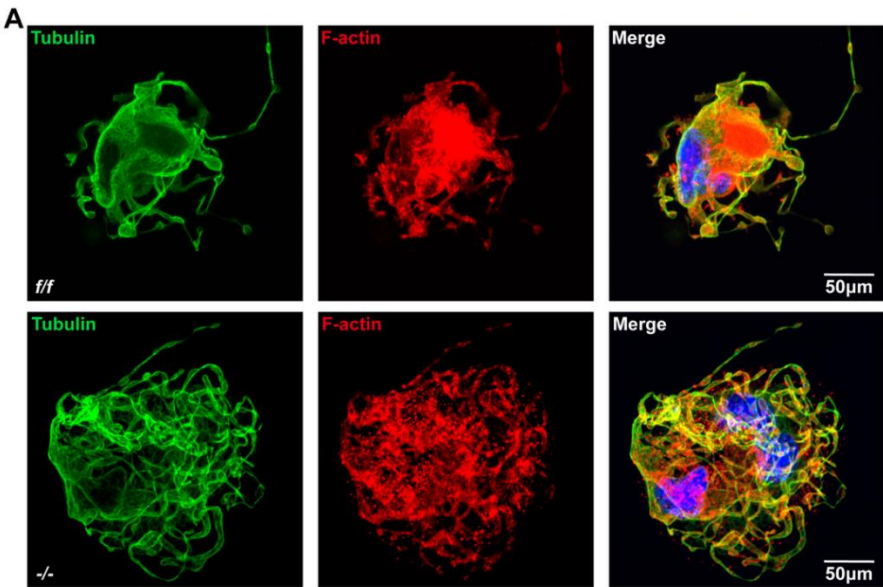
treated with NADP⁺) spreading on immobilized fibrinogen. Statistical analysis of the platelet spreading areas in the images was presented (n=6, *P<0.05). Actin cytoskeleton in platelets was stained with rhodamine-labeled phalloidin. Scale bars represented 2 μ m.

5. Some results sometimes seem a little inconsistent and may undermine the reliability of the general conclusions:

a) Fig. 2E vs Fig. 3A, we do not have the same view of the proplatelets at all; in Figure 2E they are thin, while in 3A they are very large and misshaped

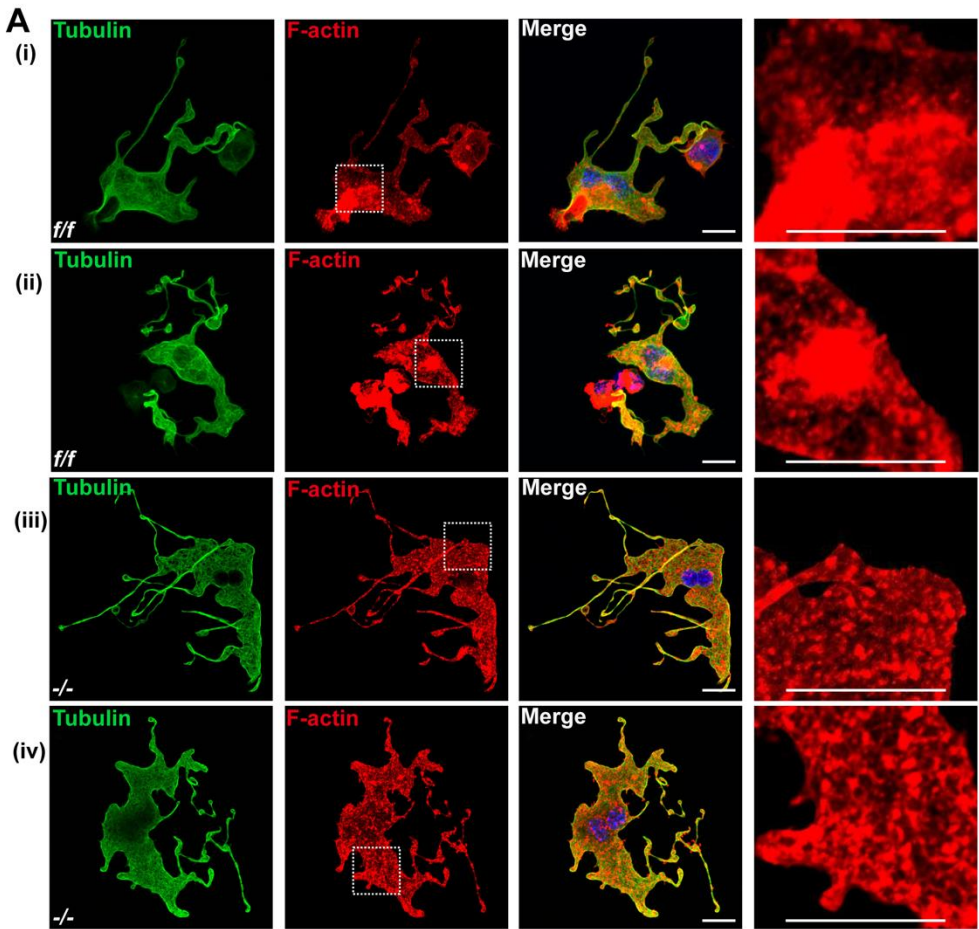
We thank the reviewer for pointing out the discrepancy between the proplatelet images shown in original Fig. 2E and original Fig. 3A. We have carefully considered this observation and identified two main factors that may have contributed to the perceived differences. First, the original images were presented at different magnifications, which could create visual heterogeneity. Second, and more importantly, the megakaryocytes shown in these two figures represent distinct stages of proplatelet formation. In original Fig. 2E, we selected megakaryocytes at a late proplatelet-formation (PPF) stage, characterized by minimal residual cytoplasm in the cell body and nearly complete nuclear extrusion, to best illustrate the counting of proplatelet tips. In contrast, original Fig. 3A displayed megakaryocytes at an intermediate stage of proplatelet formation, where the cell body still retains some cytoplasm and the nucleus is not fully extruded, allowing visualization of F-actin architecture during active proplatelet extension. Moreover, the proplatelet protrusions in the original Fig. 3A overlapped considerably, which several reviewers found confusing. To address this, we have replaced the representative image in Fig. 3A with one that better reflects the intermediate stage without overlapping protrusions, and we have also adjusted the scale to match that of Fig. 2E for consistency. The original and revised images are shown below for comparison. We believe these modifications improve the clarity and interpretability of the figures and thank the reviewer for helping us enhance the presentation of our data.

490 Before modification (Fig. 3A):



491

492 After modification (Fig. 3A):



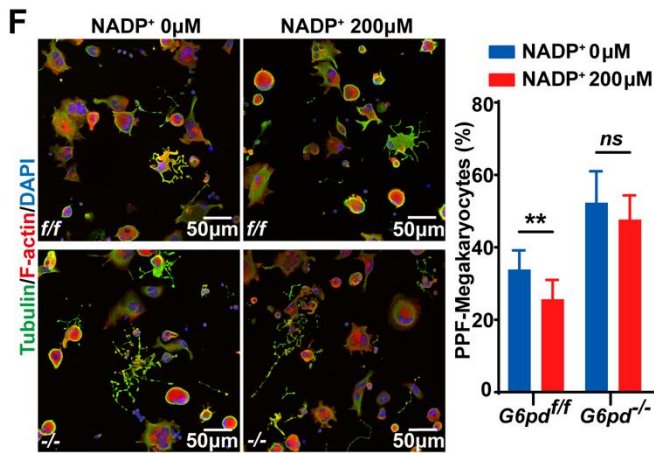
493

Fig. 3A The cytoskeleton morphology, including tubulin(green) and F-actin(red), of $G6pd^{ff}$ (i, ii) and $G6pd^{-/-}$ (iii, iv) MKs in PPF stage were observed under ZEISS 880 confocal microscope. Scale bars represented 20 μm .

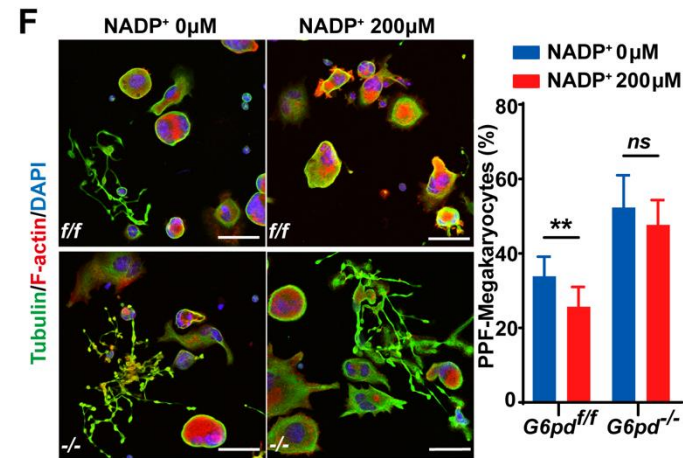
b) Fig. 6F: data vs quantification

We thank the reviewer for the careful examination. Regarding this point, we confirm that the quantitative result is robust and unaffected. Therefore, we have selected and replaced the images with more representative ones to better illustrate the findings.

Before modification:



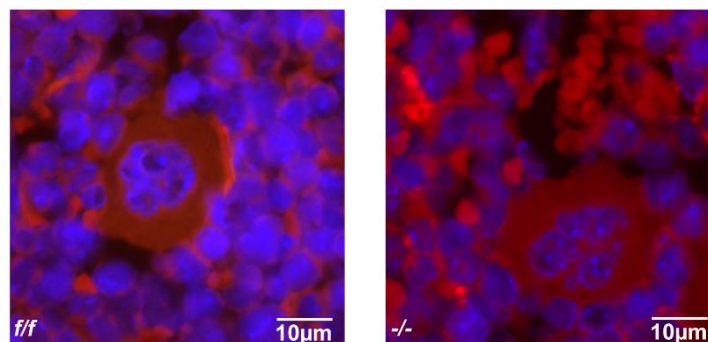
After modification:



c) *Figure 2B suggests that G6pd^{-/-} megakaryocytes exhibit ectopic platelet release. There is pink staining in the medullary space. Could you confirm this using immunofluorescence or TEM?*

To address this question, we attempted immunofluorescence staining on mouse femoral bone marrow sections using a CD42b antibody kindly provided by another laboratory. Unfortunately, the experiment was unsuccessful, as the CD42b antibody exhibited nearly complete non-specific labeling. Nevertheless, from the non-specific staining we were still able to identify the outlines of megakaryocytes, and within these outlines we did not observe evidence of ectopic proplatelet release (images in Fig. R13 provided below for reference). Furthermore, a study (Markus Spindler et al, 2022; PMID:36251748) indicated that ectopic platelet release is generally associated with defective F-actin organization and reduced platelet counts, which contrasts with the phenotype observed in our study. However, we fully recognize that these observations do not constitute definitive evidence. Therefore, we plan to further investigate this question using alternative antibodies and transmission electron microscopy of mouse bone marrow. If the reviewer remains interested in this issue, we would be happy to share our validated results once they become available; please feel free to leave your email address for future correspondence. We appreciate the reviewer's insightful comment, which has encouraged us to explore this important aspect more thoroughly.

Fig. R13



d) *Could you provide the number of elements counted per field? Indeed, it is noted $n = x$, but*

how many cells are there per field; for platelets, proplatelets, MK/mm², etc.? This information would provide a clearer statistical representation.

To provide a clearer statistical representation, we have now quantified the number of megakaryocytes per unit area in the proplatelet formation assays and the number of platelets per unit area in the spreading assays from the fluorescence images. As shown in Fig. R14A (new Fig. 2G; page 8, lines 144-145 in the revised manuscript) and Fig. R14B, our analysis revealed no significant differences between control and G6PD-deficient groups in either megakaryocyte or platelet density per field, indicating that G6PD deficiency does not affect the adhesive capacity of megakaryocytes or platelets.

Fig. R14

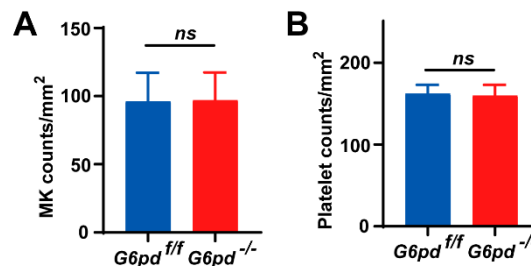


Fig. R14 (A) Cell count/mm² of MKs under the microscopy in the PPF assay (n=6). (B) Cell count/mm² of platelets under the microscopy in the platelet spreading assay (n=5).

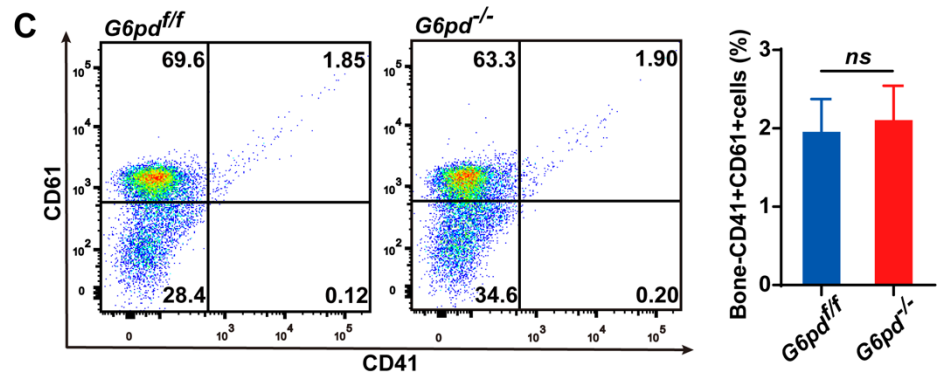
e) I do not understand the analysis in Figure 2C. Could you outline the full gating strategy? Could you also explain why more than 60% of the elements are CD41+ rather than CD42+?

We sincerely thank the reviewer for this critical observation. We regret that an error was made during the initial data processing: the gating threshold for CD61 positivity was incorrectly set, and this mistake was not identified during our subsequent review. To address this, we have re-analyzed the raw data, replaced the image with the corrected version and added the gate strategy to the new version.

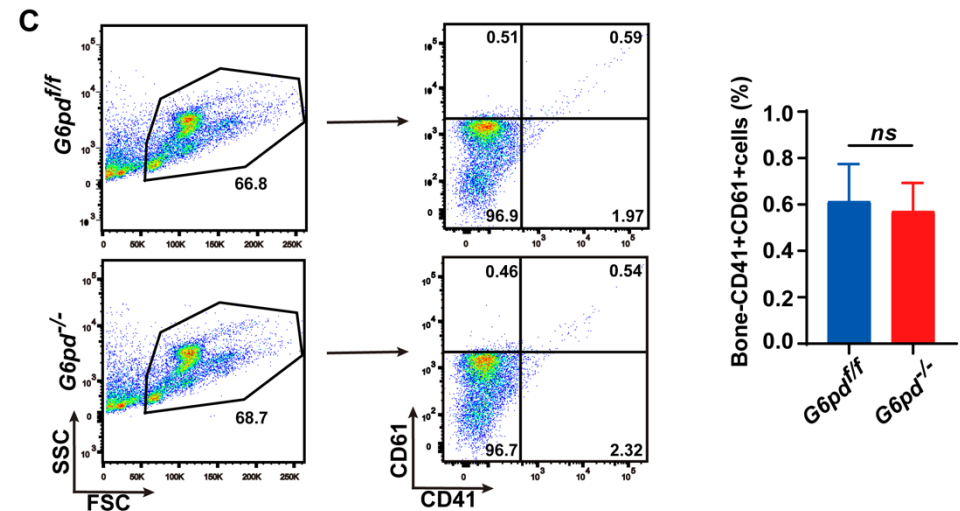
We apologize for this oversight and appreciate the reviewer's careful scrutiny, which has helped

us significantly improve the accuracy and clarity of our manuscript.

Before modification (Fig, 3C):



After modification (Fig, 3C):



Minor comments :

1. Authors should explain the observed populations in Fig. 2C
2. Could there be any possible effects of elevated NADP⁺ independent of G6PD-TMOD3 axis, as Fig. 6F suggests a decreasing trend for the KO line.

Upon careful re-analysis of the dataset, we found that NADP⁺ treatment reduced the proportion of PPF-MKs by 24% in the WT group compared to controls (34.52±4.636 vs 26.33±4.68, p=0.0048), whereas the reduction was only 9% in the KO line. Most importantly, statistical analysis revealed no significant difference within the KO line (52.98±8.062 vs 48.32±6.062,

p=0.2454). Therefore, we concluded that in the absence of G6PD, NADP⁺ did not exert a significant effect on proplatelet formation, and the inhibitory effect of NADP⁺ on PPF was dependent on the G6PD-TMOD3 axis.

3. Figure 2H was mentioned several times, but the submitted manuscript does not contain such panel.

We apologize for this error and appreciate the reviewer's careful reading again. We have now corrected the citation.

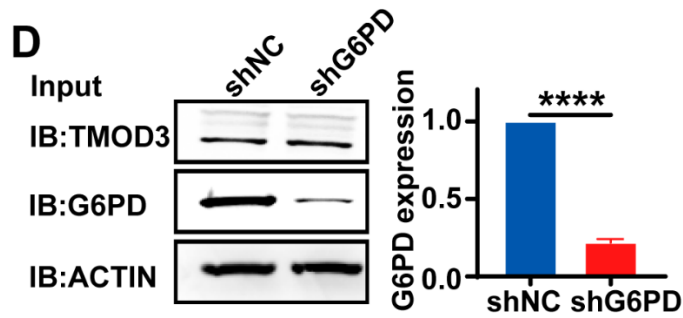
4. Taking in account differences in the number of reticulated platelets that are known to be larger, could the differences in spreading area in Fig. 3B be the result of initial larger size of platelets?

The proportion of reticulated platelets, which are known to be larger, was extremely low in both groups (Figure 1D of the revised manuscript; 0.97 ± 0.29 vs 1.39 ± 0.25); and measurements of mean platelet volume by hematology analyzer revealed no significant difference between G6PD-deficient and control platelets (Figure EV1A of the revised manuscript). Therefore, we conclude that the increased spreading area observed in G6PD-deficient platelets is attributable to enhanced actin cytoskeletal remodeling capacity rather than to an initially larger platelet size. We appreciate the reviewer's insightful comment, which has helped us clarify this point.

5. Fig. 5D needs quantification to support the conclusions

We thank the reviewer for this valuable suggestion. In response, we have now added the quantitative analysis to support the conclusions in Fig. 5D of the revised manuscript (n=3).

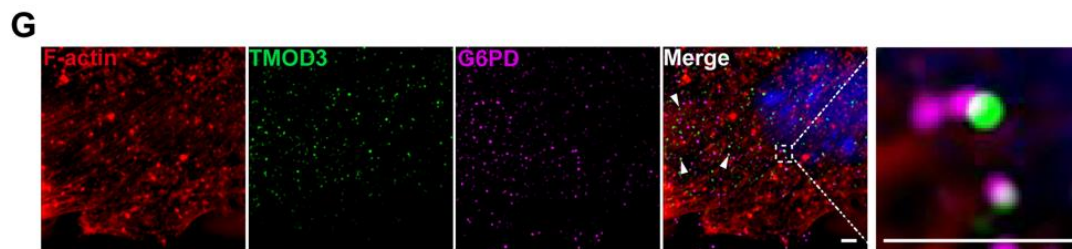
After modification (Fig. 5D):



6. Figure 5G would benefit from zoom image as in Fig. 5H and Fig. 6C

We thank the reviewer for this helpful suggestion. In response, we have now added a zoomed-in image to Fig. 5G to better illustrate the details, similar to the presentation in Fig. 5H and Fig. 6C.

After modification (Fig. 5G):



7. Figure 5H suggest polarization of the TMOD3 signal in *G6pd*^{-/-} megakaryocytes. Is it representative of the whole population? Authors should address this point

Yes, the polarized distribution of TMOD3 shown in Figure 5H is representative of the overall population of *G6pd*^{-/-} megakaryocytes. We have now explicitly stated this in the figure legend of Figure 5H and have also incorporated a description of this polarization phenomenon into the Results section of the revised manuscript (page 11, lines 222–223).

Page 11, lines 222–223: In *G6pd*^{-/-} MKs at the pre-proplatelet stage, the distribution of TMOD3 exhibited a certain degree of polarized localization (Fig. 5H.I).

8. Add a figure number to each one

We thank the reviewer for this helpful suggestion. For figures containing multiple panels, we have now added corresponding subfigure numbers.

Referee #3:

In their paper "G6PD suppresses thrombopoiesis via interfering with TMOD3-mediated actin dynamics", Xin et al. describe a novel, non-canonical function of G6PD in platelet formation. The authors demonstrate interactions between G6PD and TMOD3, thereby regulating actin dynamics. Their investigation combines multiple approaches: a G6PD knockout mouse model, several complementary cell line models using transfection or transduction, and a pharmacological strategy with a peptide. Notably, the study is introduced with patient data, which strengthens the clinical relevance of the topic. Overall, the article is of great interest, although additional experiments are required to consolidate some of the conclusions.

My detailed comments are provided below, following the order of the manuscript:

Page 5, line 81: please correct to G6PD-deficient.

We thank the reviewer for such careful proofreading. We have corrected the wrong word "G6PD-dificent" to "G6PD-deficient" as suggested (page 5, line 86 in the revised manuscript).

Page 6:

In Table 1, the statistical analyses performed should be described.

As described in the Methods section, all statistical analyses performed in this manuscript, including those for Table 1, were conducted using unpaired two-tailed Student's t-tests. We have

now ensured that this information is clearly stated in the revised manuscript (page 32, lines 629–632). We appreciate the reviewer's careful attention to detail.

Page 32, lines 629–632: Unpaired two-tailed Student's *t* test was used to analyze the results and a significance level of $p < 0.05$ was considered to determine statistically significant variations among the values. Values were represented as mean $\pm$ standard deviation.

For platelet count experiments, the sex and age of mice should be indicated.

We thank the reviewer for this suggestion. In response, we have now added this information to the figure legend in the revised manuscript (page 37, lines 793–794).

Page 37, lines 793–794: All mice were males and aged 8–10 weeks.

Platelet aggregation and secretion assays should be repeated with different agonist concentrations to confirm the absence of effect of G6PD deletion.

To further confirm the effect of G6PD deletion on platelet function, we have repeated the platelet aggregation assays using an additional concentration of thrombin and an additional concentration of collagen. As shown in Fig. R15A (Fig. EV1B of the revised manuscript), G6PD deficiency did not significantly affect platelet aggregation capacity at any of the agonist concentrations tested. Regarding the dense granule secretion assays, we found that collagen stimulation was insufficient to reliably induce secretion under our experimental conditions. Therefore, we have maintained the original results obtained with different concentrations of thrombin as shown in Fig. R15B (Fig. EV1C of the revised manuscript), which consistently showed no significant differences between genotypes. We appreciate the reviewer's insightful comment, which has helped us strengthen the rigor of our functional analyses.

Fig. R15

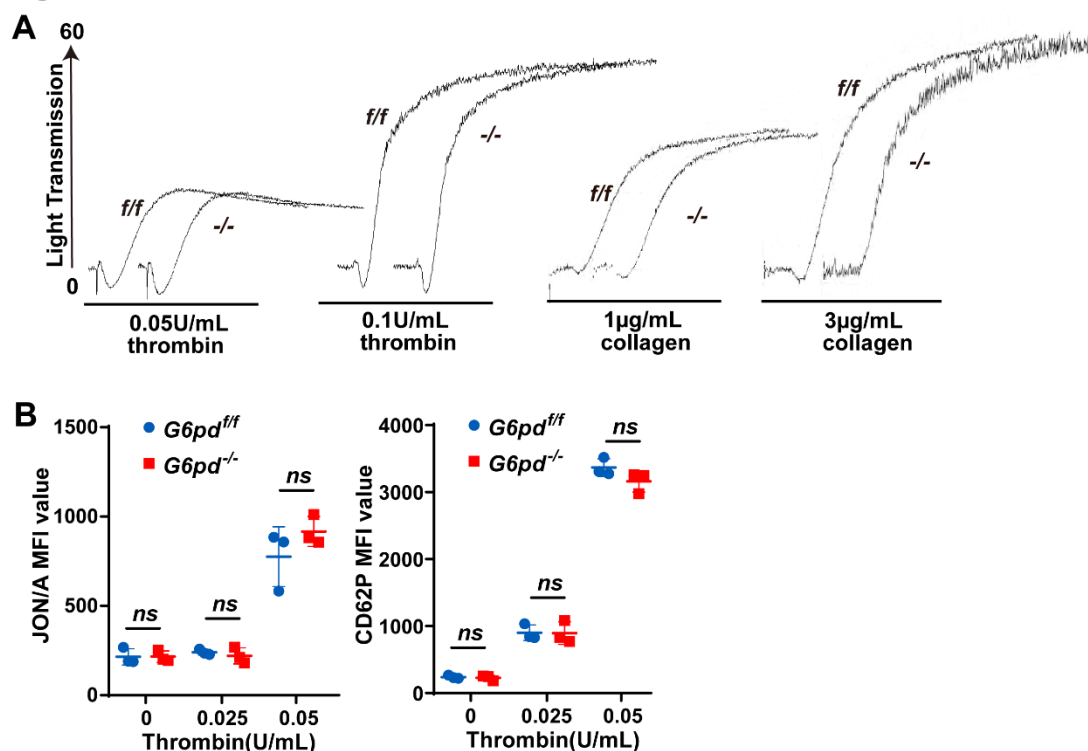


Fig. R15 (A) Aggregation of washed *G6pd^{f/f}* and *G6pd^{-/-}* platelets in response to thrombin (0.05 U/mL, 0.1 U/mL) and collagen (1 µg/mL, 3 µg/mL). **(B)** JON/A and CD62P (P-selectin) exposure of washed *G6pd^{f/f}* and *G6pd^{-/-}* platelets stimulated with 0.025 and 0.05 U/mL thrombin (n=3).

Pages 7-8:

Line 130/137: Figure 2h is missing.

We thank the reviewer for pointing out this oversight. In the revised manuscript, we have corrected this error accordingly. We appreciate the reviewer's careful reading and valuable feedback.

Line 133: the section title should be revised. The study does not demonstrate "accelerated" actin remodeling; only time-lapse or kinetic analyses could support such a claim.

We thank the reviewer for these rigorous suggestions. We agree that the term "accelerated" implies kinetic changes that our experimental design (which did not include time-lapse or

dynamic analyses) could not definitively support. Accordingly, we have revised the section title and replaced "accelerated" with "could alter" throughout the relevant text (page 8, line 152). This modification more accurately reflects the scope of our observations.

Page 8, line 152: G6PD deficiency could alter the actin cytoskeleton remodeling.

Line 137: "could" should be used instead of a direct causal statement, as there is no definitive link between G6PD deficiency and altered actin dynamics.

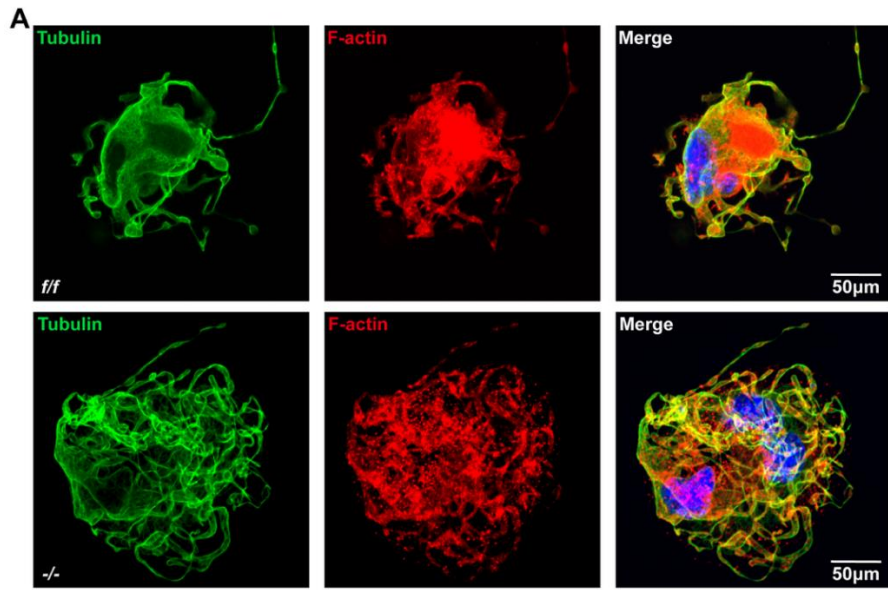
In accordance with the reviewer's suggestion, we have revised the text by incorporating "could" into the description to reflect a more appropriate level of certainty (page 8, line 157).

Page 8, line 155-157: Consequently, the finding that G6pd^{-/-} MKs at PPF-stage exhibited increased numbers of branches and proplatelet tips (Fig. 2F,I,J) strongly suggested that G6PD deletion could induce modifications in actin dynamics.

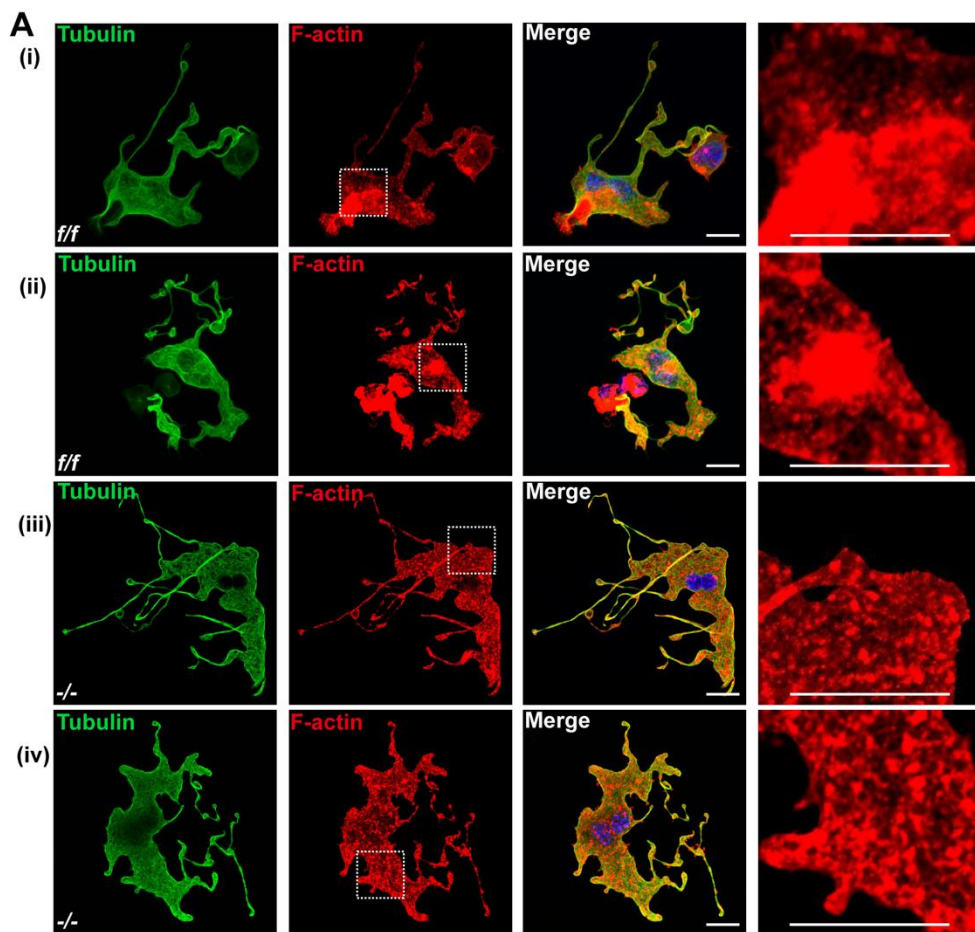
Figure 3A / line 145: quantification of the spatial distribution of F-actin is required. Measurements of F-actin polymerization should also be provided in both cell lines and platelets. Without kinetic data, the conclusion should remain cautious with respect to the suggested "acceleration."

In the original manuscript, the proplatelet-forming megakaryocytes shown in Figure 3A exhibited considerable overlap of protrusions, which may have made the F-actin morphology misleading—an issue also raised by other reviewers. Therefore, we have replaced these images with more representative ones (shown below).

Before modification (Fig. 3A):



After modification (Fig. 3A):



In early-stage proplatelet-forming megakaryocytes (prior to nuclear extrusion and retaining some cytoplasm), the accumulation of F-actin in *G6pd*^{ff} megakaryocytes and the more evenly dispersed F-actin in G6PD-deficient megakaryocytes are consistent and reproducible phenotypes. After careful consideration, we believe that these representative images sufficiently illustrate the conclusion “The images showed distinct F-actin distribution patterns between the two group: F-actin often exhibited an accumulation in specific regions of undivided cytoplasmic area in *G6pd*^{ff} MKs, while it exhibited a more uniform spatial distribution in *G6pd*^{-/-} MKs (Fig. 3A)” in the manuscript. Moreover, we have not identified a scientifically rigorous method to reliably quantify the spatial distribution of F-actin. Therefore, we consider that quantification of F-actin spatial distribution may not be necessary for this observation.

For platelets, in addition to measuring spreading area, we have now included an analysis of spreading stages to further characterize the phenotype. Platelet spreading involves multiple stages of cytoskeletal reorganization, including the formation of actin nodules for adhesion, followed by the generation of filopodia, lamellipodia, and actin filaments for area expansion (S.D.J. CALAMINUS et al, 2008; PMID: 18761725). In response, we categorized platelets into distinct stages based on whether nodules or other actin structures predominated, and the statistical results are presented in Fig. R16A shown below (new **Fig. 3B, C; page 9, lines 166-168** in the revised manuscript). The analysis revealed that within the same time frame, a significantly higher proportion of G6PD-deficient platelets had progressed to later spreading stages compared to controls, which was consistent with the observed differences in spreading area, suggesting that G6PD deficiency promoted actin organization in platelets.

For cell lines, we performed spreading assays using G6PD-knockdown Meg01 cells stimulated with PMA to assess F-actin polymerization. As presented in Fig. R16B (new **Appendix Fig. S3A; page 11, lines 206-208** in the revised manuscript), fluorescence images demonstrated significantly increased spreading area in G6PD-knockdown Meg01 cells, suggesting that G6PD deficiency also promoted actin organization in Meg01 cell line.

Furthermore, in accordance with the reviewer's suggestion, we have replaced the term "accelerated" throughout the manuscript with more cautious wording such as "enhanced" or

"altered" to avoid implying kinetic changes that were not directly measured. We appreciate the reviewer's rigorous feedback, which has helped us refine both our data presentation and scientific language.

Fig. R16

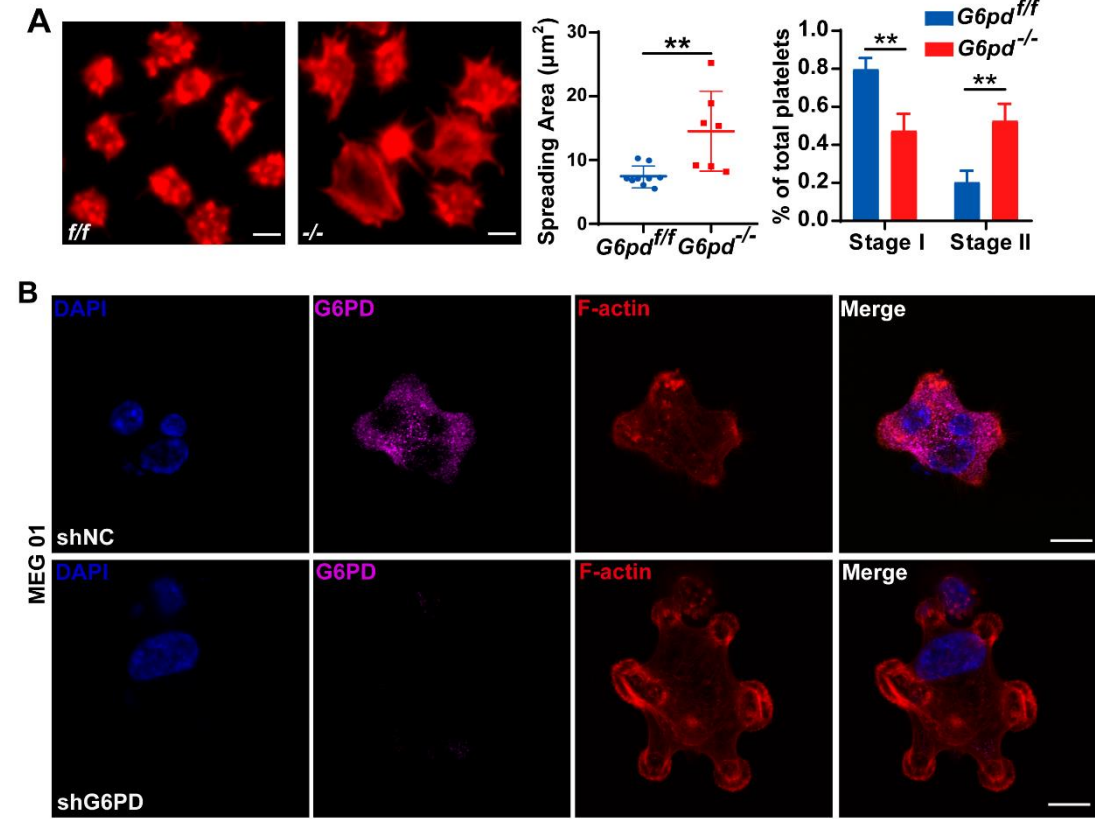


Fig. R16 (A) Representative immunofluorescence images of actin cytoskeleton in *G6pd^{f/f}* and *G6pd^{-/-}* murine platelets spreading on immobilized fibrinogen. Actin cytoskeleton in platelets was stained with rhodamine-labeled phalloidin. Statistical analysis of the platelets spreading areas from the two images was presented (n=9 vs n=7, **P<0.01). Scale bars represented 2 μm . Platelets were categorized into stages based on the predominance of nodules (Stage I) versus predominance of other actin structures (Stage II), and the percentage of platelets at different stages from three random fields captured under 63 $\times$ oil immersion objective were presented (n=3, **P<0.01). (B) G6PD-knockdown (shG6PD) and control (shNC) Meg01 cells were stimulated with Phorbol 12-myristate 13-acetate (PMA). The cells were then allowed to spread on immobilized fibrinogen for 48 hours, followed by fixation and immunofluorescence staining. Immunofluorescence images via Leica SP8X displayed the distribution of G6PD (purple) and F-actin (red). Scale bars represented 10 μm .

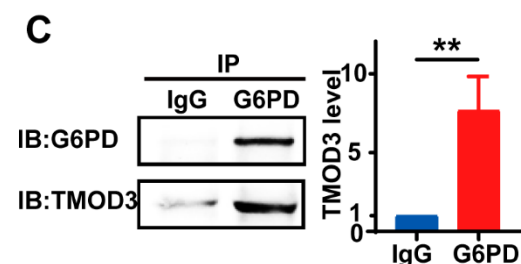
Page 9, line 163: proteomic database comparisons between Ig control and specific IgG are needed to validate signal specificity.

As presented in **Supplementary material 1**, all proteins used for subsequent pathway analysis were detected exclusively in the specific IgG group and were absent in the IgG control group, thereby ensuring the specificity of the identified G6PD-interacting proteins.

Figure 5C: quantification and n values are missing.

In response, we have now added the quantitative results and n values to **Figure 5C** in the revised manuscript (n=3, **P<0.01).

After modification (Figure 5C):



Figures 6B-6D: authors should describe how relative protein levels were calculated.

We thank the reviewer for this suggestion. We now have added the detailed description in the figure legend.

Page 41, lines 882-886 (the figure legend of Fig. 6B): Co-IP of Flag-G6PD and HA-TMOD3 in HEK 293T cells (transfected with Flag-G6pd and HA-Tmod3 plasmids) with the treatment of NADP⁺. The gray values of HA-TMOD3 bands in the IP samples were normalized against the corresponding Flag-G6PD bands. These normalized values were then calculated relative to the control (NADP 0μM) HA-TMOD3 band, and the resultant ratios were represented as the relative levels of HA-TMOD3 binding with Flag-G6PD.

Pages 41, lines 893-896 (the figure legend of Fig. 6D): The gray values of the HA-G6PD and

ACTIN bands were normalized as described in Figure 6B. The statistical analysis of relative levels of HA-G6PD binding with Flag-TMOD3 and relative levels of ACTIN binding with Flag-TMOD3 under diverse NADP⁺ concentration were presented (n=3, *P<0.05, **P<0.01, ****P<0.0001).

Peptide experiments:

A negative control (e.g., scrambled peptide) is required in the biological assays.

We thank the reviewer for this important suggestion regarding the need for an appropriate negative control in our biological assays. We fully agree that using a scrambled peptide as a control would be more rigorous. In fact, in our previous experiments, we had already included a scrambled peptide control group for most assays (with the exception of the *in-vivo* mouse platelet depletion-recovery experiment). Accordingly, in the revised manuscript, we have replaced the original control group data in **Figure 7** with the scrambled peptide control data and have indicated the scrambled peptide sequence in the figure legend. Additionally, we have repeated the *in-vivo* platelet depletion-recovery assay using the scrambled peptide as a control, and the updated results are now included in **Figure 7**.

After modification (Figure 7):

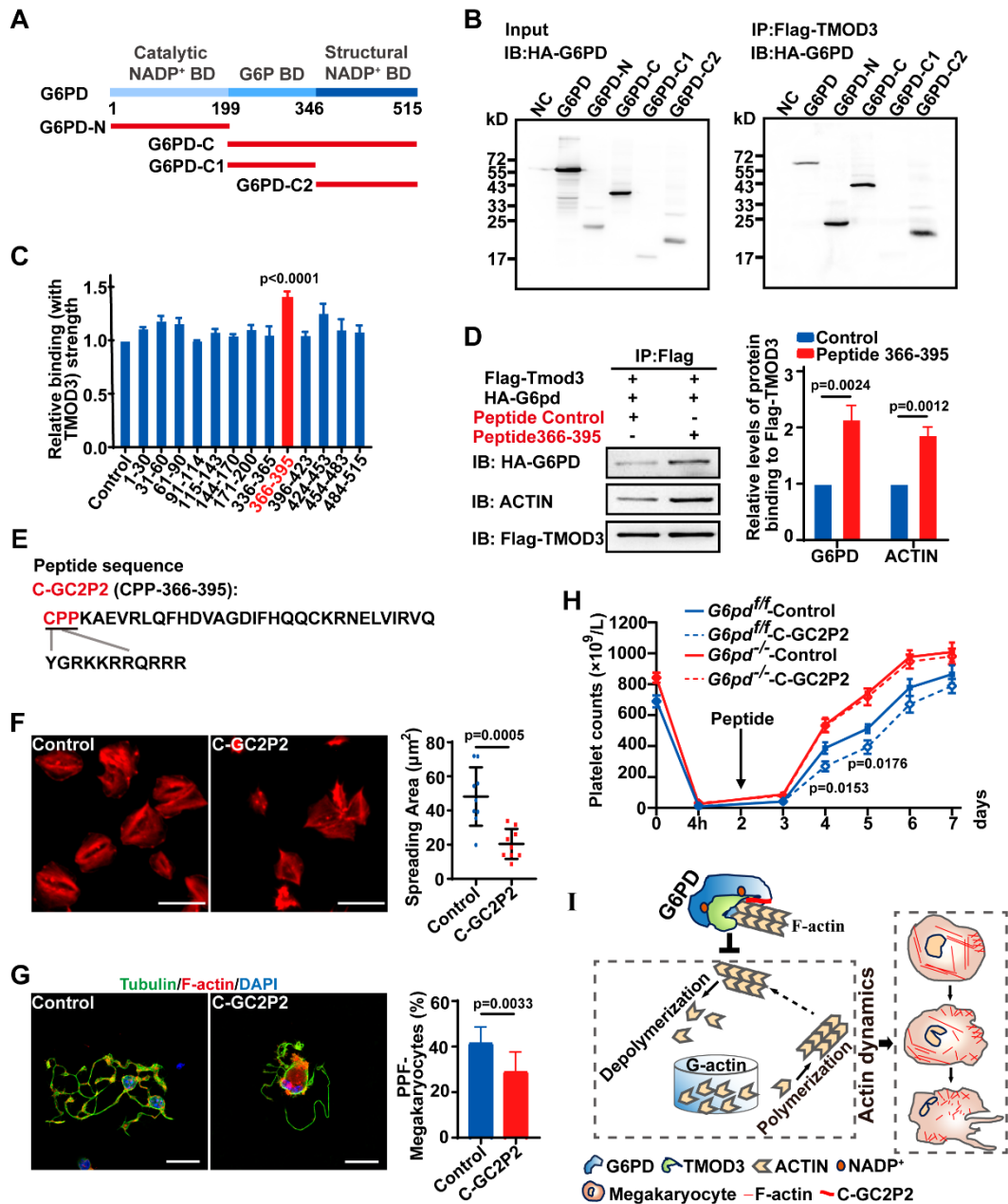


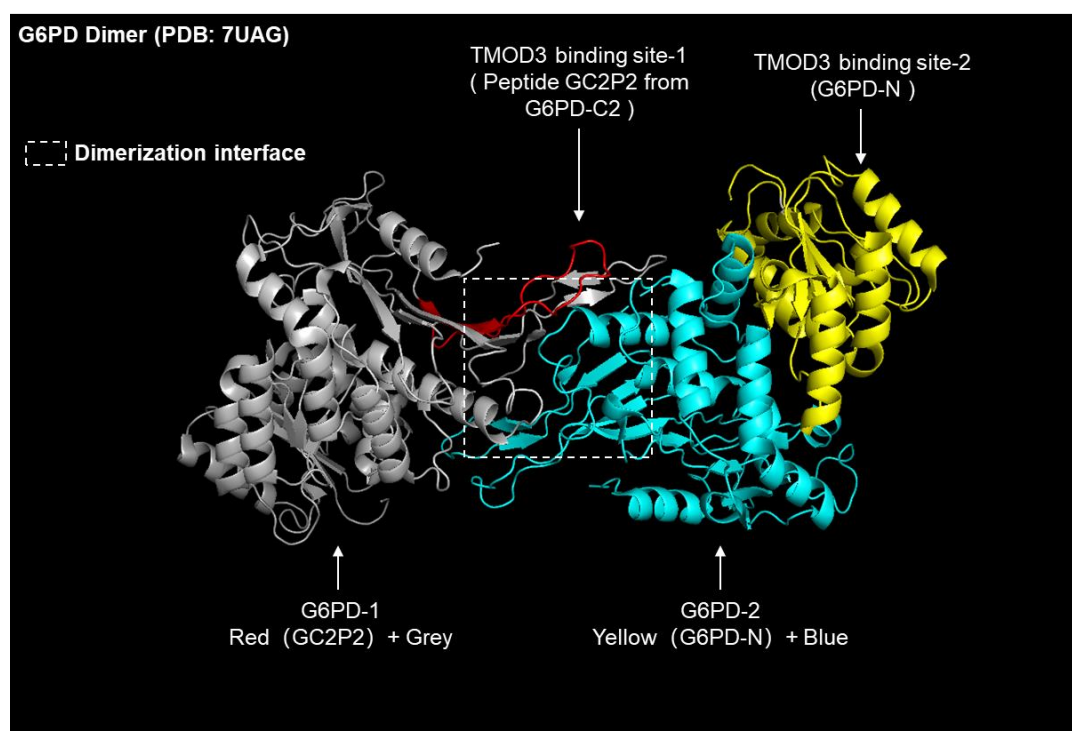
Figure 7. Peptide C-GC2P2 promoted G6PD-TMOD3 interaction and inhibited actin organization and platelet biogenesis in G6PD-dependent manner. (A) A simple schematic for functional domains as well as designed truncations of G6PD. **(B)** Co-IP of lysates from HEK293T cells transfected with Flag-Tmod3 and HA-tagged truncated forms of G6pd. **(C)** Binding affinity of thirteen G6PD peptides for TMOD3 were detected by ELISA. Statistical analysis of results from four independent experiments was shown (n=4). **(D)** Co-IP of lysates from

HEK293T cells transfected with Flag-Tmod3 and HA-G6pd. The lysate was respectively added the control peptide (Scramble; QRFHILALVQCKFQQDHDHRKVEVDVRNEGAI) and the peptide 366-395. The statistical analysis of relative levels of HA-G6PD binding with Flag-TMOD3 (the gray values of the HA-G6PD IP bands) and relative levels of ACTIN binding with Flag-TMOD3 (the gray values of the ACTIN IP bands) were presented (n=3). (E) We added TAT (YGRKKRRQRRR), a type of CPP derived from HIV, to the N-terminal of GC2P2 (366-395), constructing peptide C-GC2P2 which possessed cell-penetrated ability. (F) Immunofluorescence images of actin cytoskeleton in human platelets (treated with 50µg/mL control peptide CPP-Scramble or peptide C-GC2P2) spreading on immobilized fibrinogen. Statistical analysis of the platelet spreading areas in the images was presented (n=9). Scale bars represented 10 µm. (G) Immunofluorescence images of PPF-MKs (derived from wildtype murine fetal liver) treated with 50µg/mL control peptide CPP-Scramble or peptide C-GC2P2. Statistical analysis for the proportions of PPF-MKs counted in randomized low magnification views was presented (n=9). Scale bars represented 20 µm. (H) Bone marrow administration of the peptide C-GC2P2 or control peptide CPP-Scramble (20 µg/g weight) was carried out at 48 hours after tail intravenous injection of anti-GPIIbα antibody (1.8 µg/g weight). Platelet count was monitored at different time points. (n=3). (I) The brief mechanistic model for actin-dynamic regulatory function of G6PD in proplatelet formation and the G6PD-derived peptide C-GC2P2 was utilized to promote this function. Unpaired two-tailed Student's *t* test was used to analyze the results and a significance level of $p < 0.05$ was considered to determine statistically significant variations among the values. Values were represented as mean $\pm$ standard deviation.

It remains unclear whether the peptide promotes the interaction between G6PD and TMOD3, or instead acts as a dominant-negative inhibitor. This issue must be clarified experimentally and conceptually.

We thank the reviewer for this critical and insightful comment regarding the mechanistic role of the GC2P2 peptide. Through ELISA screening, we identified GC2P2 as the G6PD-derived peptide with the strongest binding affinity for TMOD3 (**Fig. 7C** in the revised manuscript). Subsequent co-immunoprecipitation experiments demonstrated that adding GC2P2 to protein lysates containing G6PD and TMOD3 enhanced their interaction (**Fig. 7D** in the revised manuscript). This result was unexpected, as GC2P2—being a peptide interacting with TMOD3—would conventionally be expected to act as a competitive inhibitor. Although we have not yet directly elucidated the underlying mechanism, we would like to offer a speculative explanation based on our current data for the reviewer's consideration. G6PD monomers bind structural NADP⁺ to promote dimer formation (Xuepeng Wei et al, 2022; PMID:35858355), and our results show that NADP⁺ enhances the G6PD-TMOD3 interaction (**Fig. 6B, D** in the revised manuscript). Based on these findings, we hypothesize that TMOD3 preferentially interacts with the dimeric form of wild-type G6PD. Both the N-terminal and C2 regions of G6PD appear to participate in TMOD3 binding (**Fig. 7A** in the revised manuscript), leading us to speculate that TMOD3 may simultaneously engage the N-terminus of one G6PD monomer and the C2 region of the other monomer within a dimer. Interestingly, the GC2P2 peptide from the C2 region that also overlaps with the dimerization interface. We therefore propose that GC2P2 may partially mimic the dimer interface of one G6PD monomer, effectively providing a "dimer-like surface" that can engage TMOD3 via the C2 region. Under physiological

conditions, G6PD exists predominantly as monomers; if our previous hypothesis is correct, the addition of GC2P2 could increase the availability of these "dimer-mimetic surfaces" capable of binding TMOD3, thereby enhancing the overall G6PD-TMOD3 interaction. Unfortunately, due to the lack of a stable crystal structure for TMOD3, our attempts at protein-protein docking were unsuccessful. However, we remain committed to investigating this mechanism further using alternative approaches, such as cryo-electron microscopy to resolve the protein complex structure. If the reviewer is interested in this ongoing work, we would be happy to share our progress; please feel free to leave your email address for future correspondence. We deeply appreciate the reviewer's rigorous and constructive feedback, which has challenged us to think more deeply about this unexpected finding.



Page 19, line 344: replace neonatal platelets with immature platelets, IPF, or reticulated

platelets.

Page 22, line 403: Apoptotic and reticulated platelets assays in vitro

Page 28: *the section on statistical analyses should be expanded, specifying the tests used and clarifying the graphical representations.*

Page 32, lines 629-633: Data analysis for the experiments was conducted using GraphPad Prism 8.0 software. Unpaired two-tailed Student's *t* test was used to analyze the results and a significance level of $p < 0.05$ was considered to determine statistically significant variations among the values. Values were represented as mean $\pm$ standard deviation. Moreover, all displayed images are representative images of the overall findings.

Dear Prof. Dai

Thank you for your revised manuscript. We have received two referee reports back on it. As you will see, the referees feel that all of their comments have been responded to in detail.

Referee 2 requests some further insights. However, EMBO Reports will not require that you fulfil these additional requests.

From the editorial side, there are also a few things that we need before we can proceed:

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the suggested versions below my signature.
- Please rename the "Disclosure of Conflict of Interest" section to Disclosure and Competing Interests Statement.
- Please move the Data Availability section before the Acknowledgements section.
- CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Author Contributions section from the manuscript and use the free text boxes beneath each contributing author's name in our online systems to add specific details on the author's contribution. More information is available in our guide to authors.
- Please remove the Reagents and Tools Table from the manuscript and upload it as a separate file.
- Please incorporate the cell lines used into the Reagents and Tools Table.
- Thank you for providing the Author Checklist. Please revise it to include the corresponding authors' names, the journal name, and the manuscript ID number.
- Please incorporate a statement on cell line authentication into the Methods section.
- Please expand on the mouse housing conditions in the Methods section.
- Please incorporate a statement on blinding in statistical analysis, even if no blinding was performed.
- Please provide the ORCID ID in our system for all three corresponding authors.
- Please rename the source file names for "Supplemental Material 1" to Dataset EV1.
- Please adjust the in-text callouts for individual figures and figure panels: e.g. Dataset EV1 appears to be missing.
- Please add a title page to the Appendix file with "Appendix to [Title of the ms]" and a table of contents that lists each figure and its page number.
- Thank you for providing the Source Data Checklist. Please update the entries for Fig 4A and 7A,E,I to NA (Schematic).
- EMBO Reports requires that large datasets be uploaded to a repository. Please provide direct urls in the Data Availability section.
- As I communicated previously, during a standard image analysis, we detected potential aberrations in the figure set, and we would like to clarify these issues. We kindly invite you to check the composition of:
Figure 3A & Appendix S3D - Possible cell reuse.
Appendix Figure S4B iii and iv - Possible cell reuse within figure.
Please send us any related source data. If you make changes to the figure set, please include a point-by-point describing what you have changed and why. If purposeful re-use of the image has occurred, please state this clearly in the figure legend.
- EMBO Reports requires that figures with $N < 5$ display individual data points. Please doublecheck your figures to ensure they comply with this requirement, particularly Fig 1B,C,D,F; 2A,D,E,G,H,I,J; 3C; 4B,C,F; 5C,D,F; 6B,D,F; 7C,D,G; EV1A,D,E; EV2B,G,H,J,K; S6C,D; S7A.
- Our production/data editors have asked you to clarify several points in the figure legends (see below). Please incorporate these changes in the manuscript and return the revised file with tracked changes with your final manuscript submission.
- Please note that the dotted lines are not defined in the legend of figure 5H. This needs to be rectified.

- Please note that the arrow heads are not defined in the legend of figure 6C. This needs to be rectified.

- Please note that EMBO press papers are accompanied online by:

A) a short (2 sentences) summary of the findings and their significance,

B) 2-5 short bullet points highlighting the key results, and

C) a synopsis image in .jpg or .png format that is exactly 550 pixels wide and 300 pixels high. Please note that the text needs to be legible at the final size. Please upload this information along with your revised manuscript (the text for A and B should be provided in one separate Word file uploaded as Cover Art/Synopsis Image).

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Kurt Weir
Editor
EMBO Reports

G6PD suppresses thrombopoiesis via interfering with TMOD3-mediated actin dynamics

Glucose-6-phosphate dehydrogenase (G6PD), the committed enzyme of the pentose phosphate pathway, plays a pivotal role in glucose metabolism, which is integral to platelet biology. Although G6PD deficiency is a prevalent enzyme defect worldwide, the specific role of G6PD itself in platelets remains unclear. Herein, we discover a previously unrecognized phenotype in both human patients and murine models, demonstrating that G6PD deficiency leads to elevated platelet counts through enhanced proplatelet formation (PPF). Mechanistically, we reveal a non-canonical function of G6PD in regulating actin dynamics via its interaction with an actin-binding protein, tropomodulin-3 (TMOD3). The G6PD-TMOD3 interaction, which is concentration-dependently enhanced by nicotinamide adenine dinucleotide phosphate (NADP⁺), can limit actin rearrangement, thereby inhibiting PPF. Furthermore, we develop a G6PD-derived peptide, GC2P2, which potentiates the G6PD-TMOD3 interaction and significantly suppresses thrombocytopoiesis both in vitro and in vivo. In conclusion, our findings suggest that G6PD deficiency facilitates platelet biogenesis by altering TMOD3-mediated actin dynamics. The study provides new insights into thrombopoiesis and offers potential strategies for modulating platelet production.

Referee #2:

I would like to thank the authors for their considerable work; all my comments have been addressed in detail.

However, I still have a few questions. Firstly, why was the differentiation of haematopoietic progenitor cells into proplatelet-forming megakaryocytes carried out using cells derived from the fetal liver rather than progenitors derived from bone marrow? It would be particularly informative to compare the two, given that platelets are normally produced from bone marrow progenitors and differences in the microenvironment can impact megakaryopoiesis.

Additionally, analysing megakaryocyte maturation in situ within the bone marrow using transmission microscopy, rather than only in liquid culture, would be very interesting. In bone sections, the maturation stages are easily distinguishable, as is the extension of the demarcation membrane system (DMS). In liquid culture, however, the lack of confinement often leads to a much less developed DMS and I find it difficult to draw a conclusion. Such an approach would also help address the possibility of ectopic proplatelet release within the marrow.

Finally, it is surprising that the anti-CD42b antibody did not work. What about an anti-CD42c antibody? Usually, these markers work well. That said, what about the expression of the GPIbVIX complex on the surface of MK and platelets?

Referee #3:

Authors respond to all of my concerns. Congratulations.

Reviewer 2

We sincerely thank the reviewer for the careful evaluation of our manuscript and for the constructive and insightful comments. We are pleased that our previous comments have been addressed satisfactorily. We greatly appreciate the reviewer's suggestions regarding the source of hematopoietic progenitors, the analysis of megakaryocyte maturation *in situ*, and the expression of the GPIb-V-IX complex. We have addressed these points as follows.

1. Regarding the use of fetal liver-derived rather than bone marrow-derived hematopoietic progenitors

We fully agree with the reviewer that the tissue origin of hematopoietic progenitors may influence megakaryopoiesis and proplatelet formation, and that a comparison between fetal liver- and bone marrow-derived progenitors would be informative.

We did consider using bone marrow-derived progenitors and indeed performed preliminary experiments with bone marrow-derived megakaryocytes. However, we ultimately used fetal liver-derived hematopoietic progenitors for the experiments presented in this study because fetal liver cells provide a substantially higher yield and purity of megakaryocyte progenitors and allow a more robust and reproducible generation of mature megakaryocytes and proplatelet-forming megakaryocytes *in vitro*. Fetal liver-derived progenitors are also a well-established and widely used system for studying megakaryocyte differentiation and proplatelet formation *in vitro*.

Importantly, our purpose in using this system was to establish a controlled *in vitro* model to directly assess the effect of G6PD deficiency on megakaryocyte differentiation and proplatelet formation, rather than to recapitulate all aspects of the bone marrow microenvironment.

Nevertheless, we fully appreciate the reviewer's concern that platelet/megakaryocyte-specific G6PD deficiency could potentially alter the bone marrow microenvironment *in vivo*, which may in turn influence megakaryopoiesis and platelet production. We agree that a direct comparison of bone marrow- and fetal liver-derived progenitors, together with further investigation of the bone marrow microenvironment, would be valuable.

2. Regarding the analysis of megakaryocyte maturation and the DMS *in situ*

We appreciate this important suggestion. We agree that transmission electron microscopy of megakaryocytes within the bone marrow would provide valuable information regarding their

maturation state, DMS development, and the possibility of proplatelet formation or platelet release within the marrow niche.

In the present study, we chose to examine megakaryocyte maturation and proplatelet formation primarily using the established in vitro culture system, which allowed us to more directly and quantitatively assess proplatelet formation under controlled conditions. The use of cultured megakaryocytes was also consistent with our experimental focus on the cell-intrinsic effects of G6PD deficiency on the cytoskeletal processes underlying proplatelet formation.

We agree, however, that the absence of the physical confinement and three-dimensional microenvironment of the bone marrow may affect DMS development in cultured megakaryocytes. Therefore, we acknowledge that ultrastructural analysis of megakaryocytes in situ would provide complementary evidence and could further strengthen the conclusions regarding megakaryocyte maturation and platelet biogenesis.

We thank the reviewer for this suggestion and will incorporate bone marrow-based ultrastructural analysis into our future investigations.

3. Regarding the anti-CD42b antibody and the GPIb-V-IX complex

We sincerely apologize that the anti-CD42b antibody did not provide satisfactory staining in our tissue immunofluorescence experiments. We agree that CD42c could provide a useful alternative marker, and we also appreciate the reviewer's suggestion to examine the expression of the GPIb-V-IX complex on the surface of megakaryocytes and platelets.

At the time of these experiments, we did not have a suitable anti-CD42c antibody available in our laboratory, and we were also unable to obtain an appropriate antibody within a suitable timeframe. Therefore, we did not perform the corresponding additional staining experiments. If the reviewer considers this experiment essential for the evaluation of the manuscript, we would be very happy to obtain a suitable antibody and perform the experiment as an additional validation.

Importantly, we have independently examined the surface expression of the GPIb-V-IX complex by flow cytometry in multiple experiments. We found no significant difference in the surface expression of the GPIb-V-IX complex on either megakaryocytes or platelets between G6PD-deficient and control mice. These results indicate that the increased platelet production

observed in the G6PD-deficient mice is unlikely to be attributable to altered surface expression of the GPIb-V-IX complex.

We thank the reviewer again for these thoughtful suggestions, which have helped us better define the strengths and limitations of our experimental system and will be valuable for guiding our future studies.

Prof. Jing Dai
Ruijin Hospital, Shanghai Jiao Tong University School of Medicine
Department of Clinical Laboratory Medicine
China

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